J Chem Ecol (2007) 33:1
DOI 10.1007/s10886-006-9131-y

ERRATUM

Toxicity of Aflatoxin B1 to *Helicoverpa zea* and Bioactivation by Cytochrome P450 Monooxygenases

Ren Sen Zeng · Guodong Niu · Zhimou Wen · Mary A. Schuler · May R. Berenbaum

Published online: 19 July 2006
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The first author's name is spelled incorrectly in the HTML version of the article, in the online article abstract page, and in the online table of contents for Vol. 32, No. 7: "Ren Sen L. Zeng" should be "Ren Sen Zeng" without the "L.".

The online version of the original article can be found at: http://dx.doi.org/10.1007/s10886-006-9062-7

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J Chem Ecol (2007) 33:3
DOI 10.1007/s10886-006-9132-x

ERRATUM

Pheromone-Induced Priming of a Defensive Response in Western Flower Thrips

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Published online: 19 July 2006
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The online version of the original article can be found at: http://dx.doi.org/10.1007/s10886-006-9092-1

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J Chem Ecol (2007) 33:5–24
DOI 10.1007/s10886-006-9201-1

Sequestration of Plant-Derived Phenolglucosides by Larvae of the Leaf Beetle *Chrysomela lapponica*: Thioglucosides as Mechanistic Probes

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Received: 25 August 2005 / Revised: 23 May 2006 / Accepted: 25 May 2006 /
Published online: 2 November 2006
© Springer Science + Business Media, Inc. 2006

Abstract Feeding larvae of *Chrysomela lapponica* (Coleoptera: Chrysomelidae) acquire characteristic *O*-glucosides from the leaves of their food plants. The glucosides are selectively channeled from the gut to the defensive gland. Subsequent enzymatic transformations generate a blend of different defensive compounds, e.g., salicylaldehyde and two series of 2-methylbutyl and isobutyryl esters. By using systematically modified and hydrolysis-resistant thioglucosides as structural mimics of the plant-derived glucosides, e.g., salicin and its *o*-, *m*-, and *p*-isomers 1, 2, and 3; *o*-, *m*-, and *p*-cresols 5, 6, 7; along with thioglucosides of 2-phenylethanol 9 and (3*Z*)-hexenol 10, we demonstrated that the larvae of *C. lapponica* are able to sequester a broad range of structurally different thioglucosides with comparable efficiency. This sharply contrasts with the sequestration habitus previously observed in *Chrysomela populi* and *Phratora vitellinae*, which secrete almost pure salicylaldehyde and posses a highly specific transport mechanism for salicin (Kuhn et al., Proc. Natl. Acad. Sci. USA 101:13808–13813, 2004). Also, neither *C. lapponica* nor *C. populi*

Electronic Supplementary Material Supplementary material is available at http://dx.doi.org./10.1007/s10886-006-9201-1 and is accessible for authorized users.

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sequester in their gland the thioglucoside of 8-hydroxygeraniol, the mimic of the glucoside specifically transported by larvae secreting iridoid monoterpenes (*Phaedon cochleariae*, *Gastrophysa viridula*). Accordingly, leaf beetle larvae possess selective membrane carriers in their gut and their defensive systems that match the orientation of the functional groups of glucosides from their food plants probably by embedding the substrate in a network of hydrogen bonds inside the membrane carriers. The synthesis and the spectroscopic properties of the test compounds along with a comparative evaluation of the transport capabilities of larvae of *C. populi* and *C. lapponica* are described.

Keywords Sequestration · *Chrysomela lapponica* · Transport proteins · Salicin · Defensive secretion · Salicylaldehyde

Introduction

Sequestration of plant toxins or pretoxins is widespread among insects (pyrrolizidines: Hartmann and Witte, 1995; cardenolides: Malcolm and Brower, 1989; iridoid glycosides: Dyer and Bowers, 1996; cyanogenic glycosides: Nahrstedt, 1996; glucosinolates: Nishida, 2002), but only little is known on the functional molecular basis of the uptake process. Leaf beetles offer excellent opportunities to explore this process, as sequestered compounds are often stored and concentrated in easily accessible reservoirs, in which final transformations occur. Larvae of chrysomeline leaf beetles possess pairs of dorsal defensive glands in the mesothorax and metathorax and the abdominal segments one to seven. When the larvae are disturbed, the reservoirs are everted by blood pressure and small droplets of a defensive secretion appear on top. As soon as the danger is over, the reservoirs are pulled back within the body cavity by muscle fibers and the remaining secretion is sucked back into the reservoir. Although this basic structure is fairly constant across taxa (Hollande, 1911; Garb, 1915; Paterson, 1930; Berti, 1968), the secreted toxins vary both in structure and biosynthetic origin (Fig. 1).

Several species secrete iridoid monoterpenes, e.g., chrysomelidial (Meinwald et al., 1977; Blum et al., 1978; Pasteels et al., 1982; Lorenz et al., 1993; Veith et al., 1994), which are generally synthesized *de novo* by the insect (Oldham et al., 1996; Søe et al., 2004). Species that specialize on Salicaceae, i.e., *P. vitellinae* and *Chrysomela* spp., derive salicylaldehyde from plant phenolglucosides, such as salicin and salicortin (Pavan, 1953, Pasteels et al., 1983). Particularly diverse is the defensive chemistry of the larvae of *Chrysomela lapponica* feeding on willow and poplar. Prominent series of compounds are isobutyrates or 2-methylbutyrates of 2-phenylethanol, (3*Z*)-hexenol, or benzylalkohol, respectively (Hilker and Schulz, 1994; Schulz et al., 1997; Termonia and Pasteels, 1999). The advantage of using plant-derived, glycosidically bound precursors is obvious. The compounds are often abundant in the leaves of the food plant, they are mostly non toxic *per se*, but can be readily cleaved into toxic aglycons or compounds that can be converted into toxic principles by common enzymatic activities. The glycosides are polar precursors that cannot pass membranes without the involvement of functional transport systems, and leaf beetle larvae have evolved selective transport systems that allow a channeling of certain plant-derived glucosides through the gut membrane into the defensive gland via hemolymph transport. For example, larvae of *Chrysomela populi* and *Phratora vitellinae* specifically import salicin (Feld et al., 2001; Kuhn et al., 2004). Final transformations of the glucosides into the actual defensive compounds occur within the gland (Pasteels et al., 1990; Daloze and

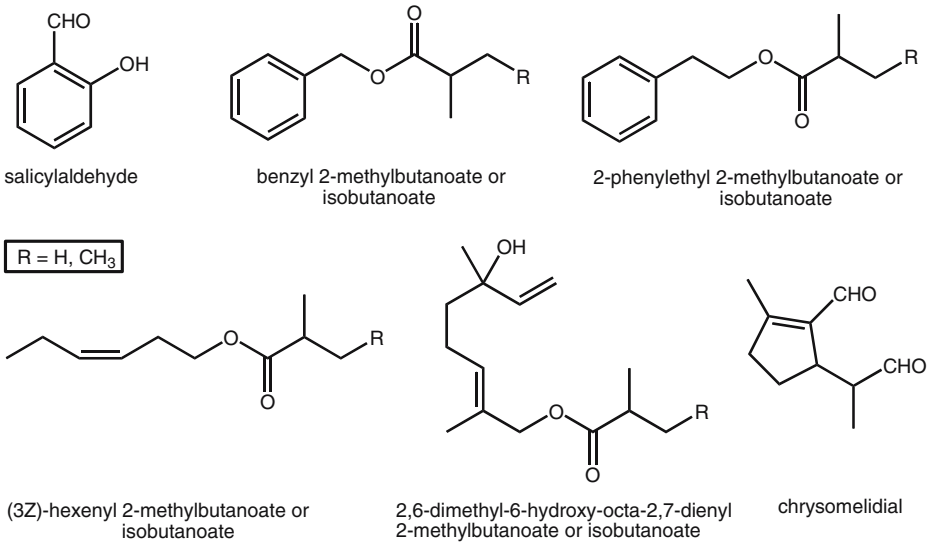


Fig. 1 Selected defense compounds from leaf beetles larvae

Pasteels, 1994; Oldham et al., 1996). As illustrated in Fig. 2, the glucosides are assumed to be selected from the food by selective carrier systems located in the gut membrane that load the glucosides into the epithelial cells, followed by export to the hemolymph. A second system shuttles the glucoside from the hemolymph to the reservoir, and here all subsequent steps of toxification can occur with strict separation from the body tissue. Besides import systems for plant-derived compounds, export systems should also exist that keep the hemolymph level of the imported molecules low to prevent an uncontrolled pretoxin accumulation in the insect body. Altogether, a network of selective and well-balanced import and export systems that facilitate the sequestration of glycosides could safely guide polar glucosides from the gut to the defensive gland.

C. lapponica is remarkable in that it is distributed into isolated populations adapted to different host plants. Larvae from Bavaria and Czech Republic feed on *Betula* and secrete complex mixtures of compounds, including series of isobutyrates and 2-methylbutyrates (Hilker and Schulz, 1994; Gross and Hilker, 1995), whereas larvae from Finland feeding on *Salix borealis*, a plant rich in phenolglucosides, have been reported to produce nearly pure salicylaldehyde (Gross and Hilker, 1995).

The current work addresses and compares the molecular basis and the selectivity of the sequestration processes in the two species *C. populi* and *C. lapponica*. Because the plant-derived *O*-glucosides are rather unstable compounds that are easily cleaved by glucosidases in the insect gut, we selected the hydrolysis-resistant *S*-glucosides instead of the natural *O*-glucosides to study the mode of their transport from the gut to the defensive gland. The glycomimics are stable against the glucosidases in the gut and the gland, and they accumulate in the reservoir where they can be easily quantified by high-performance liquid chromatography–mass spectrometry (HPLC–MS; Feld et al., 2001). We report on the synthesis of thioanalogues of salicin and several other analogues that were designed to provide information on the selectivity of the uptake of the plant-derived glucoside. Finally, we demonstrate that the larvae of *C. lapponica* possess transport systems with a higher

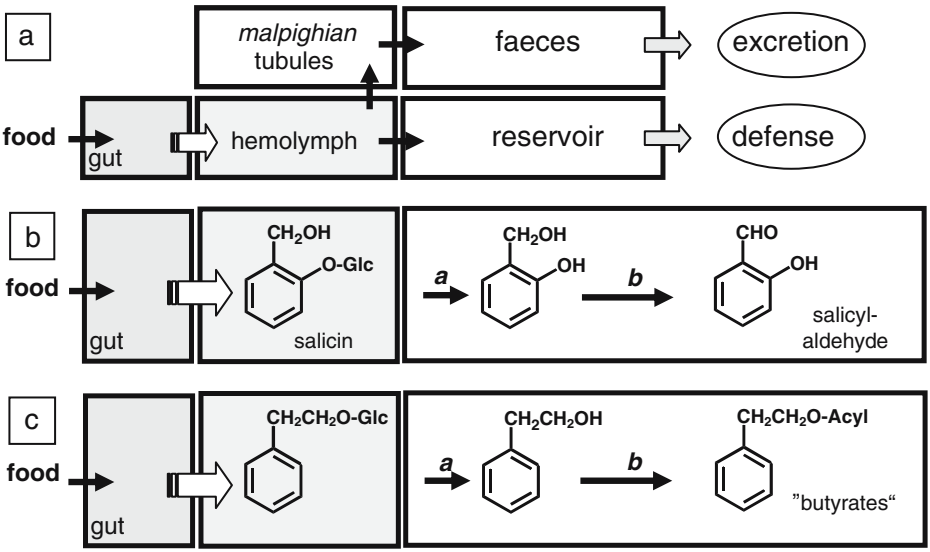


Fig. 2 (a) Transport pathways of plant-derived glucosides in the larvae of leaf beetles. Shown is the passage of glucosides from the gut into the defensive gland. At least two transport systems, from the gut to hemolymph (transport system I) and from hemolymph to the reservoir (transport system II), are necessary for sequestration. A third system may exist to excrete excess or wrong glucosides from the hemolymph to the feces. (b) Sequestration and transformation of salicin by larvae of *C. populi*. (c) Sequestration and transformation of, e.g., the glucoside of 2-phenylethanol by larvae of *C. lapponica*. After cleavage of the glucoside (a: glucosidase), the aglycon salicylalcohol (b: oxidase) is converted into salicylaldehyde (modified from Kuhn et al., 2004)

substrate tolerance, albeit with a lower overall import rate for individual compounds than those previously observed in other leaf beetle larvae.

Methods and Materials

General

Reactions were performed under Ar. Solvents were dried according to standard methods. IR: Bruker Equinox 55 FTIR Spectrophotometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR): Avance DRX 500 spectrometer; $CDCl_3$ as solvent, if not stated otherwise. Chemical shifts of ^{1}H and ^{13}C NMR are given in ppm (δ) downfield relative to TMS as internal standard. Gas chromatography (GC)–MS (70 eV): Finnigan Magnum, equipped with a fused silica capillary, coated with DB5 (30 m×0.25 mm); helium served as carrier gas. HR–MS: high-resolution GC–MS was performed on a Micromass MasSpec (Micromass, Manchester, UK) double-focusing magnetic sector mass spectrometer (geometry EBE) via solid probe. Mass spectra were measured in electron impact (EI) mode at 70 eV, with a source temperature of 200°C, an acceleration voltage of 8 kV, and a resolution of 5000. The mass range was scanned between *m*/*z* 35 and *m*/*z* 300 at 1 scan sec^{-1}. Perfluorokerosene (PFK, Aldrich, D-82041 Deisenhofen, Germany) served for calibration. Silica gel: Si 60 (0.200–0.063 mm, E. Merck, Darmstadt, Germany) was used for chromatography. Thin layer chromatography was performed with silica gel plates from Merck (60 F_{256}).

Collection and Rearing of Insects

Egg-laying *C. lapponica* were collected near Saint-Veran (Queyras, France) on *Salix breviserrata*. Neonate larvae were reared in Jena at 18°C with a photophase of 16 hr on *S. caprea* leaves until the third instar. *S. caprea* was selected because of its very low content in phenolglucoside in its leaves. As a consequence, larval secretion is not dominated by salicylaldehyde, allowing an easier detection of the other compounds.

Administration of Thioglucosides to Feeding Larvae

Leaves from *S. caprea* were painted on the upper side with 0.5 ml of MeOH/H_2O (1:1, v/v, 25 μmol ml^{-1}) containing the test compound at a concentration of 25 mM. After evaporation of the solvent, third instars (four larvae per ca. 35 cm^{-2} leaf segment) were allowed to feed on the treated leaves for 48 hr, followed by sampling of defensive secretion and hemolymph. The defensive secretion was withdrawn with a small capillary from the everted glands of four larvae. The entire operation was conducted under a binocular while the larvae were gently squeezed by tweezers to provoke extrusion of the dorsal reservoirs. To avoid contamination with material from the leaf surface adhering to the larval body, the secretion was collected only from the tip of the everted glands. Hemolymph was obtained most conveniently from larvae that were previously killed by cooling to −20°C for 30 min. The surface of the larvae was cleaned by brief dipping into water (three times), followed by clipping of a single leg. The emerging hemolymph was collected with a small capillary. The collected secretion or hemolymph was determined by weighing the capillaries before and after collection. Weighing was repeated three times to minimize errors. Defensive secretions or hemolymph were transferred into MeOH/H_2O (1:1, v/v, 40 μl). Following centrifugation, the supernatant was directly analyzed by liquid chromatography (LC)–MS.

Analysis of Thioglucosides by LC–MS

To monitor and quantify the polar thioglucosides in the defensive secretion or the hemolymph, a combination of reversed-phase HPLC and mass spectrometry was used. Analyses were carried out using a Thermoquest LCQ (Thermoquest, D-63329 Egelsbach, Germany) in the APCI mode (vaporizer temperature: 560°C) connected to an Agilent HP1100 HPLC system equipped with an RP18 column, GROM-SIL ODS-3 CP (125× 2 mm, 3 μm; Grom Chromatography GmbH, D-72108 Rottenburg-Hailfingen, Germany), and GROM-SIL ODS-7 ph (125×2 mm, 4 μm). Samples were analyzed by using gradient elution at 0.25 ml min^{-1} (solvent A: H_2O+0.5% CH_3COOH; solvent B: MeCN+0.5% CH_3COOH) according to the following protocol: starting with 2% B, then after 20 min to 100% B maintained for 8 min, and 2% B for 5 min before recycling. UV detection before the mass spectrometer allowed a convenient and reliable quantification of the thioglucosides (λ_{max}=224 nm, 254 nm). Standard curves were recorded for **1** to **11** and used for calibration (Figs. 4 and 5). Abundance was expressed as nanomoles of compound per milligram of sample (undiluted secretion or hemolymph). The threshold of detection was set to 1 nmol mg^{-1} thioglucoside in secretion. Quantification by UV was particularly valuable for the quantification of acylated thioglucosides in the defensive secretion of larvae of *C. lapponica.* Acylated thioglucosides displayed significant differences in their response based on mass spectrometry; calibration was achieved by using synthetic references (unpublished).

Statistics

Data are presented as mean values±standard deviation (Sachs, 1999). Outliers according to Grubbs test were eliminated. To obtain valid data, feeding experiments were repeated several times. *C. populi*: compounds **1** and **4** ($N=10$); **11** ($N=7$); **8** ($N=6$); **10**, **5**, and **2** ($N=5$); **9** and **3** ($N=4$); **7** and **6** ($N=3$). *C. lapponica*: compounds **10** and **9** ($N=10$); **5**, **2**, **1**, and **4** ($N=9$); **11** ($N=8$). In all experiments, *post hoc* multiple comparisons (Tamhane's T2 test, SPSS) were carried out to evaluate significant differences ($P<0.05$) between secretion samples. The hemolymph samples were not treated statistically owing to the low concentrations at the limit of the analytical approach.

Synthesis of Thioglucosides. Königs–Knorr Approach to Acetylated Aromatic Thioglucosides: General Procedure

A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.824 g, 2.0 mmol), triethylamine (560 µl, 4 mmol), and the respective aromatic thiol (2.0 mmol) in acetonitrile (4.0 ml) was stirred under argon for 2 hr at room temperature. Trichloromethane (50 ml) was added and the organic layer was thoroughly washed with water (3×10 ml). Removal of solvent ($CHCl_3$) afforded the per-acetylated thioglucoside as a light-brown oil. The product was dissolved in a small amount of warm ethanol and filtered, affording white crystals upon cooling. Spectroscopic data for the acetylated thioglucoside intermediates **12** to **21** are given in the Supplementary Material.

2-Methoxycarbonyl-phenyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**12**)

Prepared from 2-mercapto-benzoic acid methyl ester (1.5 ml, 8.9 mmol), triethylamine (3.0 ml), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (4.08 g, 9.92 mmol) as described. Yield: 4.48 (8.99 mmol, 91%).

3-Methoxycarbonyl-phenyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**13**)

Prepared from 3-mercapto-benzoic acid methyl ester (336 mg, 2.0 mmol), triethylamine (560 µl), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (824 mg, 2.0 mmol) as described. Yield: 917 mg (1.84 mmol, 93%).

4-Methoxycarbonyl-phenyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**14**)

Prepared from 4-mercapto-benzoic acid methyl ester (0.336 g, 2.0 mmol), triethylamine (560 µl) and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.824 g, 2.0 mmol) as described. Yield: 0.625 g (1.25 mmol, 63%).

Phenyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**15**)

Prepared from thiophenol (615 µl, 6.0 mmol), triethylamine (1.68 ml), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.472 g, 6.0 mmol). Yield: 2.421 g (5.5 mmol, 92%).

2-Methyl-phenyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**16**)

Prepared from 2-methyl-benzenethiol (0.242 g, 2.0 mmol), triethylamine (560 µl), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.824 g, 2.0 mmol). Yield: 0.772 g (1.7 mmol, 85%).

3-Methyl-phenyl -2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**17**)

Prepared from 3-methyl-benzenethiol (0.248 g, 2.0 mmol), triethylamine (560 µl), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.824 g, 2.0 mmol) as described. Yield: 0.757 g (1.67 mmol, 83%).

4-Methyl-phenyl -2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**18**)

Prepared from 4-methyl-benzenethiol (0.246 g, 2.0 mmol), triethylamine (560 µl), and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (0.824 g, 2.0 mmol) as described. Yield: 0.764 g (1.68 mmol, 84%).

2-Methoxycarbonyl-phenyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-galactopyranoside (**19**)

Prepared from 2-mercapto-benzoic acid methyl ester (300 µl, 2.0 mmol), triethylamine (560 µl), and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (824 mg, 2 mmol) as described. The crude product was purified instantly by flash-chromatography (flash silica 32–63 µm, 60 Å pore size), using a solvent mixture of petroleum ether (100–0%) and diethyl ether (0–100%). The product eluted at a solvent ratio of ca 1:4 and was monitored by TLC (silica gel 60, F254). Yield: 0.499 g (1.0 mmol, 50%).

Mitsunobu-Type Synthesis of Acetylated Thioglucosides **20** and **21**: General Procedure

To a solution of carefully dried ADDP (0.504 g, 2.0 mmol) in dry tetrahydrofurane (40 ml) was added with cooling (0°C) a solution of trimethylphosphane (2.0 ml, 1 M in tetrahydrofurane). The mixture was stirred at room temperature until the color turned from orange to light yellow. Then, 2,3,4,6-tetra-*O*-acetyl-thio-α-D-glucopyranose (0.472 g, 1.3 mmol) and a solution of the respective aglycon (1.0 mmol) in tetrahydrofurane (20 ml) were added with stirring at room temperature. A white precipitate occurred and after 2 hr the solvent was removed. The residue was dissolved in the minimum amount of ethyl acetate and the hydrazide was precipitated with petroleum ether. Solids were removed by filtration and the solvent was evaporated under reduced pressure. The crude product was dissolved in dichloromethane (50 ml) and washed with water (2×25 ml) and a saturated solution of $NaHCO_3$ (25 ml) solution. The product was purified by flash chromatography on silica gel using a binary solvent mixture (ethyl acetate/petroleum ether, 1:1) for elution.

2-Phenethyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**20**)

Prepared from 2,3,4,6-tetra-*O*-acetyl-thio-α-D-glucopyranose (1.42 g, 3.9 mmol) and 2-phenylethanol (0.366 g, 3.0 mmol) as described. Yield: 0.37 g (1.65 mmol, 55%).

(3Z)-Hex-3-enyl-2',3',4',6'-tetra-O-acetyl-1'-thio-β-D-glucopyranoside (**21**)

Prepared from 2,3,4,6-tetra-*O*-acetyl-thio-α-D-glucopyranose (0.472 g, 1.29 mmol) and (3*Z*)-hex-3-en-1-ol (0.10 g, 1.0 mmol) as described. Yield: 0.366 g (0.82 mmol, 82%).

Reductive Deprotection of the Thioglucosides **12**, **13**, and **14**: General Procedure

A solution of the acetylated thioglucosides **12**, **13**, or **14** (1.0 mmol) in dry tetrahydrofurane (15 ml) was added slowly to a cooled and well stirred suspension of $LiAlH_4$ [0.12 g, 3.2 mmol in the same solvent (15 ml) kept under argon]. The reaction mixture was heated to reflux for 2 hr. After cooling (0°C), excess $LiAlH_4$ was hydrolyzed by sequential addition of water (0.15 ml), 15% $NaOH_{(aq)}$ (0.15 ml), and water (0.45 ml). The resulting solid was removed by filtration. After removal of the solvent, the crude thioglucoside was purified by medium pressure liquid chromatography (MPLC) on reversed phase (RP 18 LiChroprep 40–63 μm; column 3.2 cm ID×23 cm length) using a solvent gradient for elution. For elution, a binary mixture of water (60%) and methanol (40%) was ramped within 20 min to pure methanol. Flow rate: 35 ml min^{-1} at 20 bar. The elution of products was monitored by UV at 254 nm.

2-Hydroxymethyl-phenyl-1'-thio-β-D-glucopyranoside (**1**)

Prepared from **12** (0.50 g, 1.0 mmol) by reduction with $LiAlH_4$ (0.120 g, 3.16 mmol) as described. Yield: 0.147 g (0.49 mmol, 50%). M.p.: 199.1°C. UV λ_{max} [nm]: 205 ^{1}H NMR (CD_3OD) δ [ppm]: 7.74 (dd, *J*=7.6, 1.3, 1H), 7.46 (dd, *J*=7.6, 1H), 7.32 (td, *J*=7.5, 1.4, 1H), 7.27 (td, *J*=7.5, 1.5, 1H), 4.88 (d, *J*=12.8, 1H), 4.74 (d, *J*=12.8, 1H), 4.49 (d, *J*=9.8, 1H), 3.85 (dd, *J*=11.9, 2.1, 1H), 3.65 (dd, *J*=12.2, 5.5, 1H), 3.36 (dd, *J*=8.7, 1H), 3.30–3.22 (m, 2H), 3.16 (dd, *J*=9.6, 8.7). ^{13}C NMR (CD_3OD) δ [ppm]: 144.9, 135.8, 132.8, 129.8, 129.5, 129.2, 89.8, 82.2, 79.7, 73.8, 71.5, 63.8, 63.0. IR (KBr) ν [cm^{-1}]: >3200, 3100, 3063, 3052, 2943, 2887, 1587, 1473, 1459, 1445, 1429, 1365, 1332, 1246, 1213, 1180, 1103, 1086, 1070, 1037, 1000, 751. MS (70 eV) *m/z* (%): 302(6); 145(25); 140(82); 123(40); 122 (100); 121(51); 87(28); 85(42); 77(33); 73(41); 57(29). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_6S$ 303.082410; found 303.082832.

3-Hydroxymethyl-phenyl-1'-thio-β-D-glucopyranoside (**2**)

Prepared from **13** (0.50 g, 1.0 mmol) by reduction with $LiAlH_4$ (0.120 g, 3.16 mmol) as described. Yield: 0.119 g (0.40 mmol, 40%). UV λ_{max} [nm]: 201, 251. ^{1}H NMR (D_2O) δ [ppm]: 7.60 (s, 1H), 7.55 (d, *J*=7.8, 1H), 7.30 (dd, *J*=7.9, 7.7, 1H), 7.24 (d, *J*=7.8, 1H), 4.69 (d, *J*=9.8, 1H), 4.52 (s, 2H), 3.78 (dd, *J*=12.5, 2.1, 1H), 3.60 (dd, *J*=12.5, 5.8, 1H), 3.42 (t, *J*=9.2, 1H), 3.38 (ddd, *J*=9.8, 6.0, 2.1, 1H), 3.30 (t, *J*=9.4, 1H), 3.24 (t, *J*=9.4, 1H). ^{13}C NMR (D_2O) δ [ppm]: 142.7, 133.6, 131.9, 131.4, 130.7, 128.2, 88.4, 81.2, 78.5, 73.0, 70.6, 64.7, 62.1. IR (KBr) ν [cm^{-1}]: >3100, 2925, 2853, 1269, 1120, 1016. MS (70 eV) *m/z* (%): 302(2), 162 (6), 145(14), 141(8), 140(100), 139(5), 127(9), 123(7), 109 (5), 107(7), 97(5), 91(9), 87(14), 85(20), 79(7), 77(11), 73(17). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_6S$ 302.082410; found 302.082123.

4-Hydroxymethyl-phenyl-1'-thio-β-D-glucopyranoside (**3**)

Prepared from **14** (0.475 g, 0.95 mmol) by reduction with $LiAlH_4$ (0.120 g, 3.16 mmol) as described. Yield: 0.165 g (0.55 mmol, 57%). M.p.: 77°C. UV λ_{max} [nm]: 201, 252. ^{1}H NMR (D_2O) δ [ppm]: 7.46 (d, J=8.2, 2H), 7.27 (d, J=8.2, 2H), 4.67 (d, J=10.1, 1H), 4.52 (s, 2H), 3.78 (dd, J=12.4, 1.7, 1H), 3.60 (dd, J=12.4, 5.6, 1H), 3.41 (t, J=9.0, 1H), 3.37 (ddd, J=10.0, 5.9, 1.0, 1H), 3.29 (t, J=9.6, 1H), 3.23 (dd, J=9.5, 9.6, 1H). ^{13}C NMR (D_2O) δ [ppm]: 142.9, 134.5, 133.9, 130.8, 89.9, 82.6, 79.9, 74.4, 72.0, 66.0, 63.5. IR (KBr) ν [cm^{-1}]: >3100, 2893, 2449, 2363, 1489, 1405, 1275, 1035, 860, 800. MS (70 eV) *m/z* (%): 302(3), 145(14), 142(5), 141(8), 140(100), 139(5), 127(10), 123(9), 122(7), 111(8), 109(4), 107(13), 97(6), 91(8), 87(12), 85(21), 79(8), 78(4), 77(12), 73(17), 71(3), 69(9), 61(10), 60 (9), 57(6). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_6S$ 302.082410; found 302.082756.

2-Hydroxymethyl-phenyl-1'-thio-β-D-galactopyranoside (**4**)

Prepared from **19** (0.556 g, 1.0 mmol) by reduction with $LiAlH_4$ (0.120 g, 3.16 mmol) as described. The product was separated from the solid by 12-hr continuous extraction with diethyl ether. After removal (rotavapor) of the solvent (tetrahydrofuran), the crude thiogalactoside was purified by MPLC on reversed phase (RP 18, LiChroprep 40–63 μm; column 3.2 cm ID×23 cm length) using a mixture of water (60–0% in 20 min) and methanol (40–100% in 20 min) for elution. Flow rate: ca. 35 ml/min (ca. 20 bar). The eluting product was monitored by UV at 254 nm. Yield: 0.165 g (0.55 mmol, 55%). M.p.: 158°C. UV λ_{max} [nm]: 201, 250. ^{1}H NMR (CD_3OD) δ [ppm]: 7.78 (dd, J=7.7, 1.3, 1H), 7.49 (dd, J=7.5, 1.3, 1H), 7.35 (td, J=7.5, 1.3, 1H), 7.30 (td, J=7.5, 1.5, 1H), 4.89 (d, J= 13.1, 1H), 4.79 (d, J=12.9, 1H), 4.51 (d, J=9.8, 1H), 3.91 (dd, J=3.6, 0.8, 1H), 3.79 (dd, J=11.5, 6.8, 1H), 3.73 (dd, J=11.3, 5.2, 1H), 3.60 (t, J=9.4, 1H), 3.56 (dd, J=5.4, 1.0, 1H), 3.55 (dd, J=5.8, 0.6, 1H), 3.51 (dd, J=9.2, 3.2, 1H). ^{13}C NMR (CD_3OD) δ [ppm]: 144.8, 135.2, 133.6, 129.6, 129.2, 90.8, 80.7, 76.3, 71.1, 70.5, 63.8, 62.7. IR (KBr) ν [cm^{-1}]: >3100, 2940, 2917, 2886, 1417, 1130, 1085, 1045, 1009, 950, 860, 820, 802, 758. MS (EI) *m/z* (%): 302(15), 140(71), 123(33), 122(100), 121(41), 91(46), 85(28), 77(22),73 (55), 69(16), 61(21), 57(24). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_6S$ 303.082410; found 303.082154.

Base-Catalyzed Deacetylation of Thioglucosides **15**, **16**, **17**, and **18**: General Procedure

A solution of the acetylated thioglucosides **15**, **16**, **17**, or **18** (0.73 mmol) in methanol (10 ml) is treated with sodium methoxide (0.01 ml of a 30% soln., 3.0 mmol) and stirred for 12 hr at room temperature. After neutralization with amberlite IRC50, the product is purified by MPLC on reversed phase (RP-18, Büchi column no. 17982) using a binary solvent of MeOH/H_2O (3:2) for isocratic elution or high-speed countercurrent chromatography (Triple Coil, P.C., Inc., $CHCl_3$/MeOH/H_2O (7:13:8), S_f = 0.8).

Phenyl-1'-thio-β-D-glucopyranoside (**8**)

Prepared from **15** (2.42 g, 5.50 mmol) as described. Yield: 0.94 g (3.45 mmol, 63%). Mp: 113°C. UV λ_{max} [nm]: 204, 3. ^{1}H NMR (CD_3OD) δ [ppm]: 7.58 (dd, J=8.2, 1.2, 2H), 7.32 (dd, J=8.5, 7.0, 2H), 7.27 (tt, J=7.2, 1.1, 1H), 4.50 (d, J=9.8, 1H), 3.89 (dd, J=12.2, 2.1,

1H), 3.57 (dd, *J*=12.2, 5.3, 1H), 3.41 (t, *J*=8.7, 1H), 3.35 (ddd, *J*=9.6, 5.5, 2.1, 1H), 3.32 (t, *J*=9.3, 1H), 3.25 (dd, *J*=9.6, 8.7, 1H). ^{13}C NMR (CD_3OD) δ [ppm]: 135.5, 132.9, 130.1, 128.6, 89.6, 82.3, 79.9, 74.0, 71.6, 63.1. IR (KBr) ν [cm^{-1}]: >3100, 2972, 2931, 2900, 2528, 1582, 1482, 1438, 1384, 1271, 1123, 1045, 876, 819, 738, 687. MS (70 eV) *m/z* (%): 272(3), 145(18), 127(11), 111(10), 110(100), 109(12), 97(6), 91(7), 87(14), 85(23), 77(7), 73(18), 69(10), 66(5), 65(5), 61(11), 60(5), 61(11), 60(5),57(11). HR–MS *m/z* [M^+]: calcd. for $C_{12}H_{16}O_5S$ 272.071846; found 272.071877.

2-Methyl-phenyl-1'-thio-β-D-glucopyranoside (**5**)

Prepared from **16** (0.450 g, 0.99 mmol) as described. Yield: 0.278 g (0.97 mmol, 98). UV λ_{max} [nm]: 210,3. ^{1}H NMR (CD_3OD) δ [ppm]: 7.52 (dd, *J*=7.2, 1.7, 1H), 7.21 (dd, *J*=7.8, 1.4, 1H), 7.18 (td, *J*=7.2, 1.9, 1H), 7.15 (td, *J*=7.4, 1.8, 1H), 4.69 (d, *J*=10.1, 1H), 3.78 (dd, *J*=12.5, 2.1, 1H), 3.60 (dd, *J*=12.5, 5.5, 1H), 3.35 (t, *J*=8.7, 1H), 3.48 (ddd, *J*=9.6, 5.5, 2.1, 1H), 3.45 (d, *J*=6.1, 1H), 3.43 (dd, *J*=8.5, 7.6, 1H), 2.42 (s, 3H). ^{13}C NMR (CD_3OD) δ [ppm]: 140.0, 135.2, 132.7, 131.1, 128.2, 127.7, 89.3, 82.0, 79.7, 74.2, 71.4, 62.9, 21.1. IR (KBr) ν [cm^{-1}]: >3100, 2925, 1589, 1466, 1363, 1272, 1228, 1108, 1045, 921, 884, 825, 737, 680. MS (70 eV) *m/z* (%): 286(3), 145(12), 124(100), 97(6), 91(21), 87 (13), 85(22), 73(15), 69(9), 61(10), 57(12). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_5S$ 286.087496; found 286.087761.

3-Methyl-phenyl-1'-thio-β-D-glucopyranoside (**6**)

Prepared from **17** (0.476 g, 0.99 mmol) as described. Yield: 0.297 g (1.04 mmol, 99%). M.p.: 91°C. UV λ_{max} [nm]: 203, 250. ^{1}H NMR (CD_3OD) δ [ppm]: 7.36 (s, 1H), 7.30 (d, *J*=7.6, 1H), 7.13 (t, *J*=7.6, 1H), 7.02 (d, *J*=7.6, 1H), 4.54 (d, *J*=10.1, 1H), 3.82 (dd, *J*=12.2, 1.8, 1H), 3.62 (dd, *J*=12.2, 5.5, 1H), 3.35 (t, *J*=8.7, 1H), 3.28 (ddd, *J*=9.2, 5.9, 1.9, 1H), 3.25 (dd, *J*=9.3, 8.7, 1H), 3.18 (dd, *J*=9.8, 8.9, 1H), 2.22 (s, 3H). ^{13}C NMR (CD_3OD) δ [ppm]: 140.1, 135.3, 133.4, 130.0, 129.3, 89.7, 82.3, 79.9, 74.1, 71.7, 63.2, 21.6. IR (KBr) ν [cm^{-1}]: >3100, 2908, 1586, 1473, 1419, 1375, 1280, 1214, 1123, 1067, 890, 828, 772, 688. MS (70 eV) *m/z* (%): 286(4), 145(10), 127(8), 126(5), 125(10), 124 (100), 123(6), 97(5), 91(21), 87(10), 85(18), 73(13), 69(7), 61(9), 57(9). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_5S$ 286.087496; found 286.087410.

4-Methyl-phenyl-1'-thio-β-D-glucopyranoside (**7**)

Prepared from **18** (0.459 g, 1.01 mmol) as described. Yield: 0.274 g (0.96 mmol, 96%). M.p.: 148°C. UV λ_{max} [nm]: 201, 250. ^{1}H NMR (CD_3OD) δ [ppm]: 7.37 (d, *J*=8.2, 2H), 7.14 (d, *J*=8.2, 2H), 4.59 (d, *J*=9.8, 1H), 3.77 (dd, *J*=12.5, 2.1, 1H), 3.59 (dd, *J*=12.4, 5.6, 1H), 3.40 (t, *J*=8.9, 1H), 3.33 (ddd, *J*=9.8, 5.5, 2.1 1H), 3.27 (t, *J*=9.6, 1H), 3.21 (q, *J*= 9.0, 1H), 2.22 (s, 3H). ^{13}C NMR (CD_3OD) δ [ppm]: 139.0, 133.8, 131.4, 130.8, 89.9, 82.2, 79.9, 73.9, 71.6, 63.1, 21.4. IR (KBr) ν [cm^{-1}]: >3200, 3021, 2912, 2878, 2641, 1488, 1438, 1388, 1322, 1239, 1211, 1178, 1079, 1045, 868, 807. MS (70 eV) *m/z* (%): 286(6), 145(8), 127(6), 124(100), 123(10), 97(5), 91(22), 87(10), 85(15), 73(13), 69(7), 61(8), 57 (9). HR–MS *m/z* [M^+]: calcd. for $C_{13}H_{18}O_5S$ 286.087496; found 287.087404.

2-Phenyl-ethyl-1'-thio-β-D-glucopyranoside (**9**)

Prepared from **20** (0.773 g, 1.65 mmol) as described. Yield: 0.476 g (1.58 mmol, 96%). UV λ_{max} [nm]: 201, 259. ^{1}H NMR (CD_3OD) δ [ppm]: 7.24–7.17 (m, 4H), 7.17 (tt, J=6.8, 1.8, 1H), 4.37 (d, J=9.5, 1H), 3.87 (dd, J=11.9, 2.1 1H), 3.62 (dd, J=11.9, 5.5, 1H), 3.37–3.37 (m, 1H), 3.31 (t, J=8.7, 1H), 3.27 (ddd, J=9.5, 6.0, 2.4, 1H), 3.21 (dd, J=9.8, 8.5, 1H), 2.99–2.84 (m, 4H). ^{13}C NMR (D_2O) δ [ppm]: 141.2, 129.3, 129.2, 127.1, 85.6, 80.4, 77.8, 73.0, 70.1, 61.5, 36.2, 31.6. IR (KBr) ν [cm^{-1}]: > 3100, 3026, 2923, 1499, 1453, 1350, 1273, 1050, 885, 822, 698. MS (70 eV) m/z (%): 300(3), 282(3), 264(2), 251(2), 223(3), 163(10), 162(10), 145(58), 138(57), 127(37), 116(56), 105(100), 104(61), 91(58), 87(43), 85(69), 73(61), 69(23), 61(31), 60(30), 57(35). HR–MS m/z [M^+]: calcd. for $C_{14}H_{20}O_5S$ 300.103146; found 300.102707.

(3Z)-Hex-3-enyl-1'-thio-β-D-glucopyranoside (**10**)

Prepared from **21** (0.325 g, 0.73 mmol) as described. Yield: 0.133 g (0.48 mmol, 67%). Mp: 65°C. ^{1}H NMR (CD_3OD) δ [ppm]: 5.80–5.55 (m, 2H), 4.67 (dd, J=9.8, 4.5, 1H), 4.03 (d, J=12.5, 1H), 3.88 (dd, J=12.2, 4.3, 1H), 3.65–3.57 (m, 3H), 3.48 (td, J=9.5, 1.6, 1H), 2.94–2.91(m, 2H), 2.57–2.46 (m, 2H), 2.25–2.12 (m, 2H), 1.11 (t, J=7.5, 3H). ^{13}C NMR ($CDCl_3$) δ [ppm]: 134.2, 127.4, 85.7, 80.2, 77.7, 72.8, 69.9, 61.3, 30.2, 27.7, 20.7, 14.1. IR (KBr) ν [cm^{-1}]: 3416, 2942, 2832, 1637, 1458, 1261, 1112, 1032, 804. MS (70 eV) m/z (%): 278(2), 234(1), 229(1), 205(2), 198(4), 187(3), 169(5), 163(13), 162(18), 145(78), 127 (53), 117(18), 116(43), 115(100), 103(13), 99(14), 97(21), 91(27), 87(92), 85(90), 83(18), 82(22), 81(12), 73(65), 71(18), 69(34), 67(17), 61(39), 60(30), 57(41), 55(43). HR–MS m/z [M^+]: calcd. for $C_{12}H_{22}O_5S$ 278.118796; found 278.118609.

Results

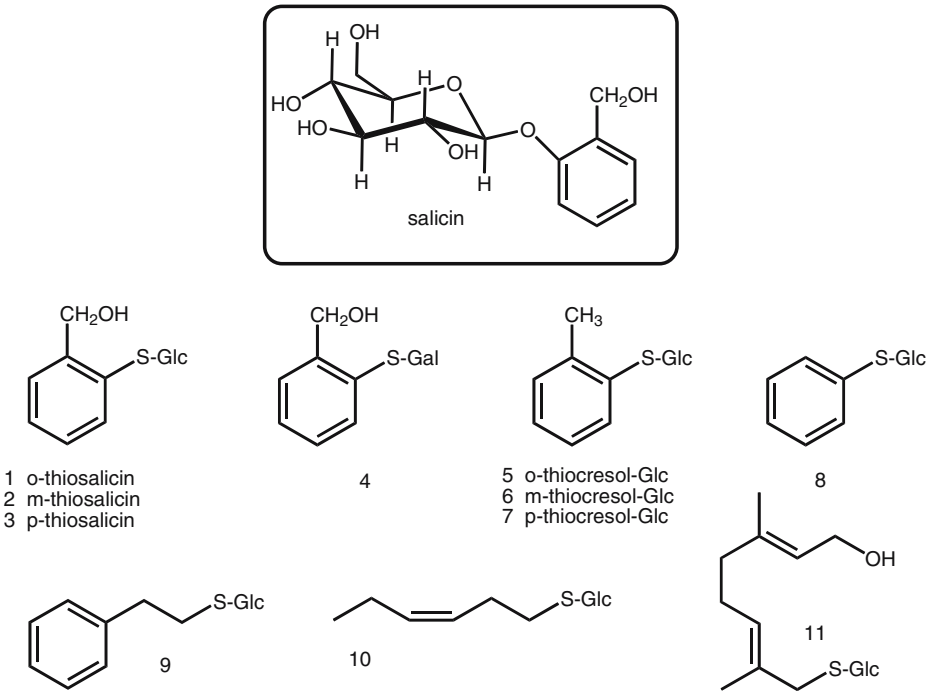
Analysis of the Defensive Secretion of *C. lapponica* Larvae Fed on *S. caprea* (Queyras, France)

Chrysomela lapponica were collected near Saint-Veran, Queyras, France, and their larvae were fed on *S. caprea*. The total ion chromatogram of their exocrine secretion is shown in Fig. 3. For comparison, the secretion of *C. populi* is shown as an insert. Although both species feed on *Salix* spp., generally rich in different phenolglucosides, only the secretion of *C. lapponica* shows a large number of compounds ultimately derived from glucosides of the host plant. A list of the identified compounds of *C. lapponica* comprising salicylaldehyde and of two series of isobutyrates and 2-methylbutyrates of 2-phenylethanol, benzylalcohol, hydroxylinalool, and (3*Z*)-hexenol is given in the legend of Fig. 3. A more detailed analysis will be reported elsewhere.

Synthesis of Thioglucosides

To understand the selection criteria for the selective sequestration of salicin by larvae of *C. populi*, and the broad range import of plant-derived glucosides by *C. lapponica* (see Fig. 3), we chose isomeric thioglucosides of salicin, and other aliphatic or aromatic

thioglucosides, which occur in different host plants, as test compounds. Thioglucosides offer a unique structural similarity to the natural substrates retaining the ability to adopt the same bioactive conformations as natural carbohydrates and conjugates (Nicotra, 1998). At the same time, they exhibit exceptional chemical and biological stability, making them to ideal tools to study transport phenomena of glucosides (Scheme 1).

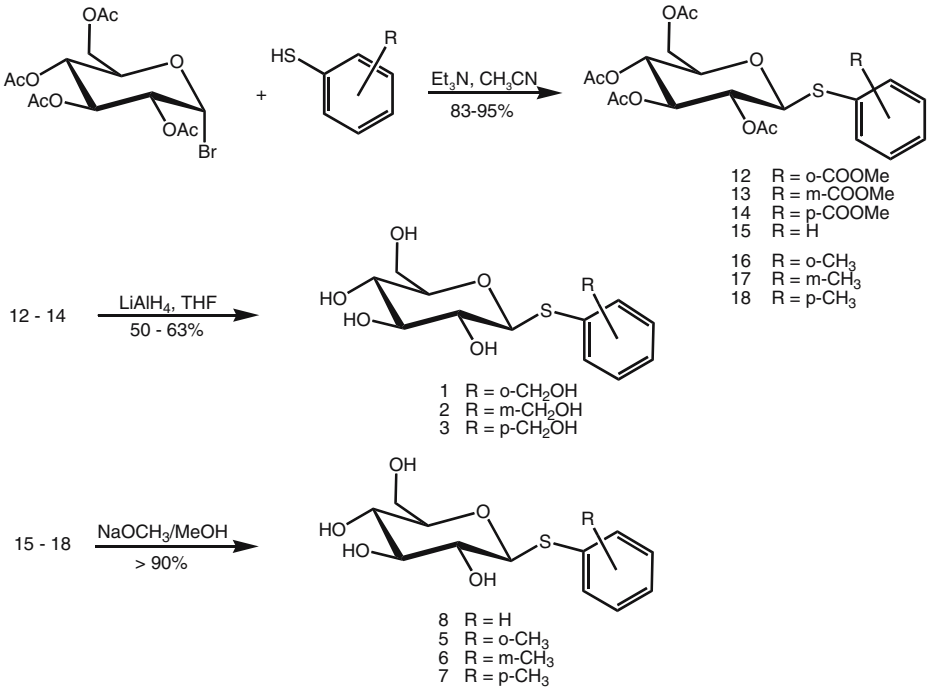


Scheme 1 Survey on thioglucosides **1** to **10** designed as structural analogues of the natural *O*-glucoside salicin from poplar leaves

The test compounds **1** to **9** cover thioglucosides of the *o*-, *m*-, and *p*-isomers of salicin, as well as those of thiocresols, which lack the hydroxyl group of the C_1 substituent of salicin. Compound **4** maintains all features of **1**, except that the glucose moiety is replaced by galactose, epimeric to glucose at C-4. The thioglucoside **8** lacks the hydroxymethyl group of salicin. Compounds **9**, **10**, and **11** represent stable *S*-analogues of natural *O*-glucosides found in *Salix* or birch leaves that are sequestered by larvae of *C. lapponica* (Table 1 in Schulz et al., 1997). Compound **11** was demonstrated to be specifically transported by larvae secreting iridoid monoterpenes (Feld et al., 2001; Kuhn et al., 2004). Compounds **1** to **11** were previously tested on *C. populi* (Kuhn et al., 2004; see Fig. 4) and the thioglucosides **1**, **2**, **4**, **6**, **9**, **10**, and **11** were used in the present study to evaluate the selectivity of the transports system(s) of *C. lapponica*.

The synthesis of most of the aromatic thioglucosides **12** to **18** followed a uniform protocol based on alkylation of acetobromoglucose with *o*-, *m*-, *p*-mercaptobenzoates or *o*-,

m-, *p*-mercaptocresols as nucleophiles (Scheme 2). The isomeric thiosalicins **1**, **2**, **3**, and **8** were obtained after reductive removal of the acetate moieties with $LiAlH_4$. The hydride reagent simultaneously reduced the benzoate ester to the required -CH_2OH substituent. The thioglucosides of the isomeric cresols **6**, **7**, and **8** were liberated from the acetylated precursors by treatment with methoxide in methanol. The basic cleavage was the method of choice, if only the acetates had to be removed, because the work-up proved to be more efficient than after reduction with $LiAlH_4$.

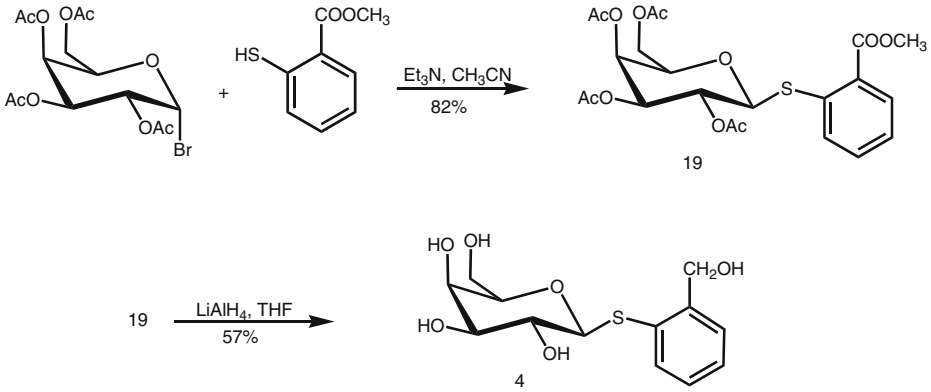


Scheme 2 Synthesis of aromatic thioglucosides via Königs-Knorr protocol

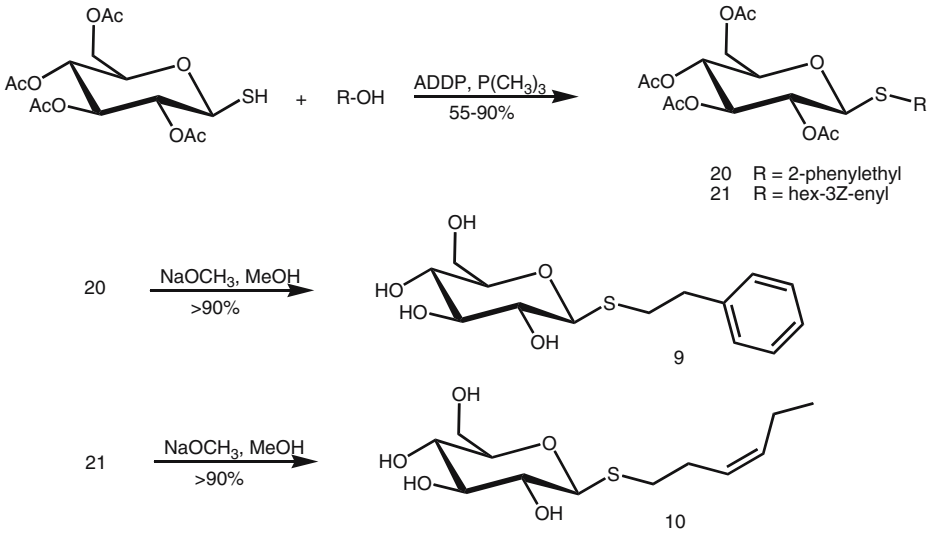
The galactoside **4** was also obtained via the Königs–Knorr protocol by using peracetylated α-bromogalactose and *o*-mercaptobenzoate as the nucleophile.

After reductive removal of the acetates with simultaneous reduction of the benzoate, the galactoside **4** was obtained in 57% yield (Scheme 3). The synthesis of the thioglucosides **9** and **10** required a different approach, because the thionucleophile corresponding to (*Z*)-hex-3-enol or phenylethanol was not readily available. In this case, the alcohols were condensed with 2,3,4,6-tetra-*O*-acetyl-β-D-thioglucopyranose according to a modified Mitsunobu protocol (Falconer et al., 1999). The acetates were saponified in high yield with sodium methoxide in methanol (Scheme 4).

Purification of all glucosides was best achieved by MPLC on reversed phase (RP 18) by using methanol/water for elution or by high-speed counter-current chromatography as described in Methods and Materials.



Scheme 3 Synthesis of aromatic thioglucosides via Mitsunobu protocol



Scheme 4 Synthesis of thioglucosides derived from (*Z*)-3-hexenol and 2-phenylethanol

Analysis of the Transport Characteristics of the Thioglucosides by Larvae of *C. lapponica* and *C. populi*

Third instars of *C. populi* were allowed to feed on leaves of *Populus canadensis* pretreated with aqueous solutions of the different thioglucoside **1** to **11**. After 48 hr, their defensive secretions and their hemolymph were collected and analyzed by LC–MS in the atmospheric pressure chemical ionization mode after minimal sample preparation (see Methods and Materials). Although the results of the feeding experiments with *C. populi* were already published (Kuhn et al., 2004), the data are repeated in Fig. 4 to allow a comparative analysis of the transport modalities of the two systems of *C. populi* and *C. lapponica*. Because all compounds were applied at the same concentration and because the leaves were consumed to a comparable extent, the accumulation of the thioglucosides in the defensive secretion can be taken as a measure for the efficiency of the transport process.

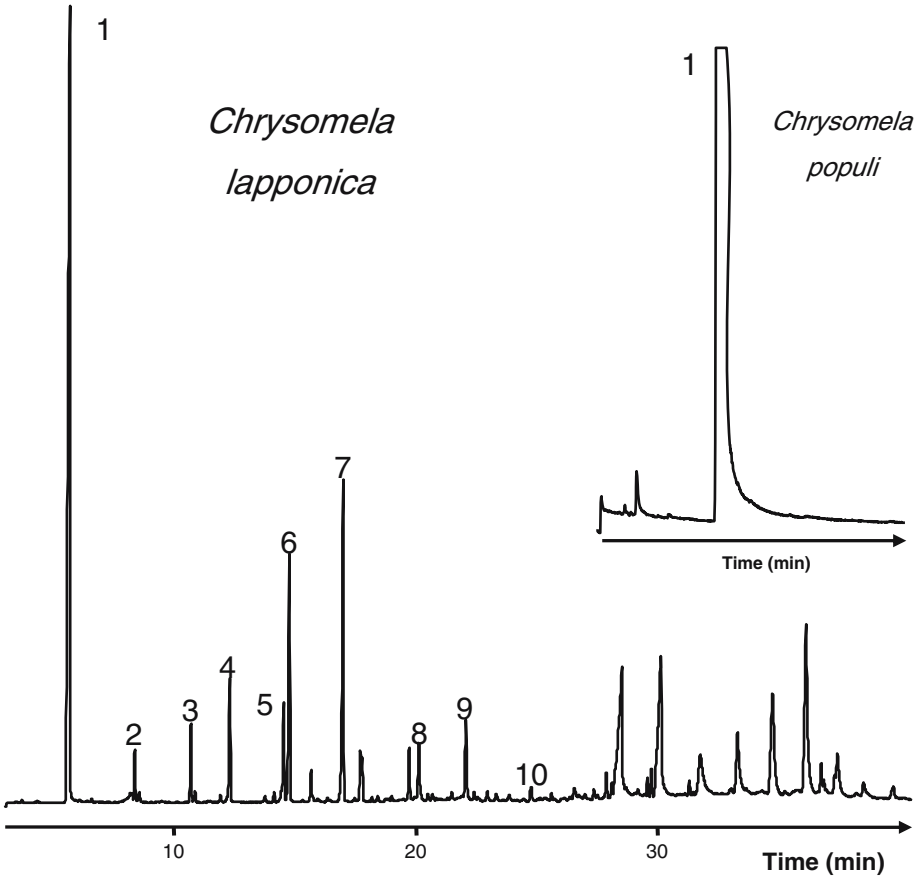


Fig. 3 Total ion chromatogram of the low boiling fraction of the exocrine defensive secretions of larvae of *C. lapponica* (Queyras, France) feeding on *S. caprea*; inset: *C. populi* feeding on *P. canadensis*. Identification of compounds: **1**, salicylaldehyde; **2**, (3*Z*)-hexenyl isobutyrate; **3**, (3*Z*)-hexenyl 2-methylbutryrate; **4**, benzyl isobutyrate; **5**, benzyl 2-methylbutyrate; **6**, 2-phenylethyl isobutyrate; **7**, 2-phenylethyl 2-methylbutyrate; **8**, 8-(isobutyryloxy)-linalool; **9**, 8-(2-methylbutyryloxy)-linalool; **10**, 2-phenylethyl benzoate

Larvae of *C. populi* sequestered only the thiosalicin **1** with high efficiency, and the compound accumulated to a final level of 1100 nmol mg^{-1} in the defensive secretion. The concentration in the hemolymph did not exceed ca. 1 to 2 nmol mg^{-1}. Unlike thiosalicin **1**, the *m*- and the *p*-isomers **2** and **3** were not notably transported and did not accumulate in the secretion (final level 10–20 nmol mg^{-1}). Surprisingly, the thioglucoside of the cresol **5**, lacking only the hydroxyl group of the salicin side chain, was also not significantly enriched in the gland reservoir. Within the limits of error, the transport efficiency was roughly the same for all three regioisomeric cresol thioglucosides **5**, **6**, and **7**, and was comparable with the transport rate of the nonnatural regioisomers of thiosalicin (**1**). The thioglucoside of 8-hydroxygeraniol **11**, the precursor for iridoid biosynthesis, was not significantly imported by larvae of *C. populi*.

When larvae of *C. lapponica* were allowed to feed on leaves of *S. caprea* pretreated with the thioglucosides **1**, **2**, **4**, **6**, **9**, **10**, or **11**, sequestration into the glandular reservoir was observed for all compounds except for the thiogalactoside **4** (Fig. 5). The latter compound

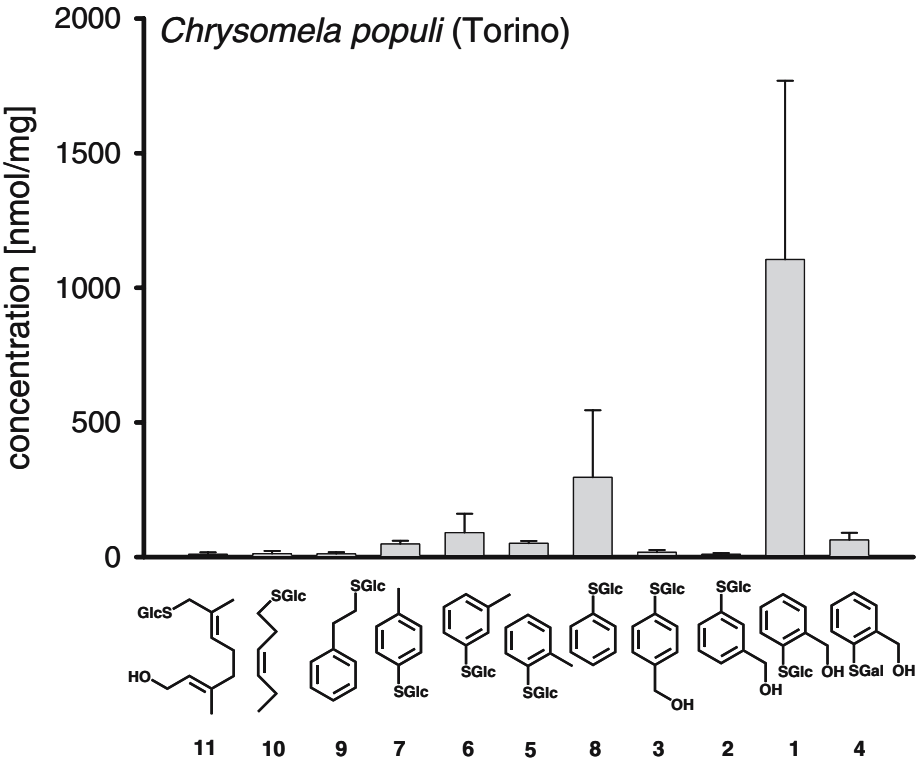


Fig. 4 Survey on the accumulation of the thioglucosides **1** to **10** in the defensive secretion of feeding larvae of *C. populi*; modified from Kuhn et al., 2004

was not imported by the feeding larvae of *C. lapponica* and only to a very low extent by other previously studied chrysomeline larvae (Kuhn et al., 2004). Consistent with the low concentration of the thioglucosides in the hemolymph of *C. populi*, in the larvae of *C. lapponica*, only a very low titer of the sequestered thioglucosides was also observed and hampered a reliable quantification. The major difference observed in *C. populi* was in the final concentrations of the imported thioglucosides in the reservoir. Although larvae of *C. populi* accumulated thiosalicin **1** to about 1100 nmol mg^{-1} in their defensive secretion, **1** and other test compounds did not exceed 200–450 nmol mg^{-1} in the secretion of *C. lapponica*. Moreover, the import pattern did not show a clear preference for a single compound. Another remarkable difference in *C. populi* was the observation that a fraction of the imported thioglucosides was acylated at the conjugating sugar or even at the alcohol moiety if additional hydroxy groups were present in the aglycon, e.g., in **1**, **2**, and **11**. The total concentration of the imported thioglucosides was, therefore, corrected for the acylated fraction by using synthetic references for calibration (unpublished data).

In larvae of *C. lapponica*, the missing hydroxyl group in the thioglucoside **6** does not affect the import of the compound; the import of **1** and **6** is achieved with similar efficiency. Moreover, the structurally unrelated thioglucosides of 2-phenylethanol **9** and (3*Z*)-hexenol **10** are also readily sequestered and accumulate to the same level as thiosalicin **1**. On the other hand, the import of the terpenoid-type thioglucoside **11** was low in larvae of *C. lapponica* from Queyras, but not from other locations (unpublished results).

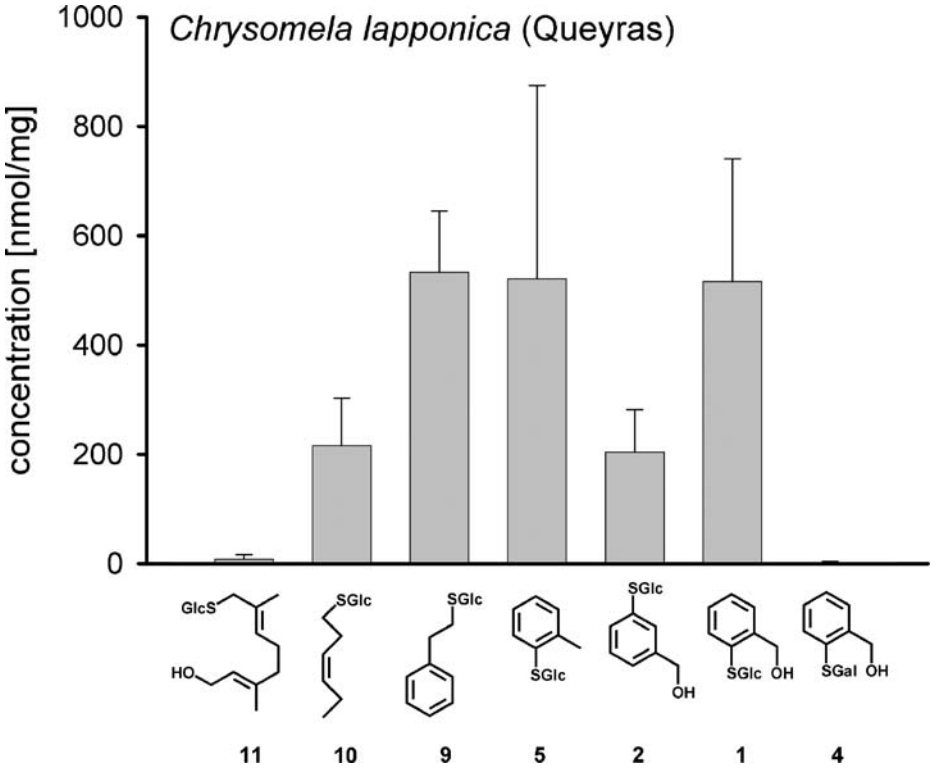


Fig. 5 Survey on the accumulation of the test compounds **1**, **2**, **4**, **5**, **9**, **10**, and **11** in the defensive secretion of feeding larvae of *C. populi*

Discussion

Selective transport systems that facilitate the sequestration of secondary metabolites from plants have been postulated or demonstrated several times (Rothschild, 1973; Blum, 1981; Hartmann, 1999). For example, transport systems are required for the uptake of pyrrolizidine-*N*-oxides from Asteraceae by *Oreina cacaliae* (Hartmann et al., 1999) or for the sequestration of the highly polar cardenolide glycosides (Duffey and Scudder, 1972). Irrespective of the enormous importance of the phenomenon of sequestration of plant-derived metabolites, none of the involved transport systems has, as yet, been identified and characterized at the molecular level. The current work, together with similar experiments with other leaf beetle larvae (Feld et al., 2001; Kuhn et al., 2004), provides the first information on the different molecular and stereochemical features that govern the uptake processes of salicin from *Populus* leaves by larvae of *C. populi*, and also other plant-derived glucosides by larvae of *C. lapponica* from Queyras (France). In both cases, thioglucosides could be successfully used as substitutes of the naturally occurring *O*-glucosides. Owing to their stability against glucosidases, which are omnipresent in the defensive secretions of leaf beetle larvae (Pasteels et al., 1990), the compounds are not cleaved during the gut passage and accumulate unchanged in the defensive gland. As illustrated in Fig. 1, a first transport system has to be located in the membrane of the gut, facilitating the uptake of the precursors into the hemolymph. A second transport system has

to be associated with the membranes of the defensive system. The latter selectively imports the glycosides from the circulating hemolymph. The concentration of the most efficiently transported glycoside **1** in the hemolymph of *C. populi* never exceeded 1–2 nmol mg^{-1}, whereas in the reservoir up to 1100 nmol mg^{-1} were measured. Comparable observations were made for *C. lapponica*, but the maximum concentrations of the thioglucosides in the reservoir were much lower (up to 450 nmol mg^{-1} secretion).

The sequestration strategy of the larvae of *C. populi* and *C. lapponica* is remarkably different. Although the import system of *C. populi* is specialized for salicin, finally converted to salicylaldehyde, larvae of *C. lapponica* import a broad range of glucosides that are converted into a blend of defensive compounds (see Fig. 5). In addition to salicylaldehyde, the secretion of *C. lapponica* contains significant amounts of isobutyl and 2-methylbutyl esters, of e.g., (3*Z*)-hexenol and 2-phenylethanol. This coincides with the observation that the thioglucosides of both alcohols are readily sequestered from food (Fig. 3). Apparently, the import of a broader range of glucosides is facilitated in these larvae by a reduced selectivity of the transport systems. This becomes particularly obvious by the comparable import rate of the thioglucosides **1**, **5**, **9**, and **10**. The lack of the hydroxyl group in the thiocresol **5** does not affect the import rate, and this may suggest that this functional group is not involved in interactions with the transport system of *C. lapponica*. This is very different in larvae of *C. populi*. Their transport system responds to this structural modification by ca. 90% reduction of the import of **5** when compared to **1** (Fig. 3). Apparently, the transport system of *C. populi* interacts with all hydroxyl groups of the substrate salicin by formation of hydrogen bridges. In the case of *C. lapponica*, the recognition of the aglycon is less strict and shows a larger tolerance to structural changes. In particular, the presence or absence of hydroxy groups within the aglycon is not critical in the case of *C. lapponica*.

A mechanistic model postulating hydrogen bridges between the functional groups of the glucoside and the transport system would also explain why the galactoside fails to be sequestered (Figs. 3 and 4). In this case, the hydroxyl group at C(4) of the sugar moiety has the wrong orientation and is not able to interact with the transport system. Because both insect species rely on the import of glucosides, this structural element is of equal importance to both. A selective interaction with the hydroxyl group of the salicin aglycon by larvae of *C. populi* also accounts for the differences between the *m*-, and *p*-isomers **2** and **3**. Because these compounds are less efficiently accumulated than the *o*-, *m*-, and *p*-cresols **5**, **6**, and **7** (Fig. 4), a hydroxyl group out of place may even prevent the correct positioning of the substrate in the transport system.

The high accumulation in the reservoir is only possible if the membrane carrier in the glandular system is coupled to an active energy source (Hartmann et al., 1999). With respect to a low hemolymph titer of **1**, the transport system in the gut must not operate against a steep gradient. Here, the concentration in the gut should be generally higher due to a constant supply of the compound from the ingested food.

An interesting aspect concerns the identity of the two required transport systems, the first in the gut and the second in the membrane of the defensive gland (see Fig. 1). The current feeding experiments give only an overall view on the selectivity of the passage of compounds through both systems. Individual analyses of the gut-associated and gland-based transport systems are needed.

Another important aspect of this work is the finding that the transport systems, once evolutionary adapted to plant-derived glucosides, apparently remain quite stable within related groups of insects. For example, larvae of the iridoid-producing *Phaedon cochleariae*, *Gastrophysa viridula*, and *Hydrothassa marginella* are able to import the

(thio)glucoside of 8-hydroxygeraniol from pretreated leaves, but fail to enrich any aromatic glucoside (Kuhn et al., 2004). Inversely, larvae of *C. populi* and *Phratora laticollis* sequester salicin, but not the glucoside of 8-hydroxygeraniol. In all cases, the high preference for a single plant-derived glucoside coincides with the composition of the defensive secretion that is based on a single or a few compounds. The more evolved *C. lapponica* apparently use transport systems of moderate selectivity that allow the import of a broader range of plant-derived glucosides. After removal of the sugar moiety, the aglycons may be oxidized (e.g., to salicylaldehyde) or acylated to give the isobutyl or 2-methylbutyl esters. Whether the import of a broad range of phytogenic glucosides is achieved by a single system with low selectivity or by a number of highly selective transport systems remains to be clarified. This and related questions on the existence of a network of interacting import and export systems within the insect have to await the isolation of the corresponding transport proteins and their genes allowing a thorough phylogenetic analysis.

Acknowledgments We thank Angelika Berg for rearing of the insects, and Dr. A. Burse for proofreading of the manuscript. Financial support by the Fonds der Chemischen Industrie, Frankfurt a.M. and from the Belgian Fund for Join Basic Research is gratefully acknowledged.

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J Chem Ecol (2007) 33:25–42
DOI 10.1007/s10886-006-9207-8

De novo Synthesis vs. Sequestration: Negatively Correlated Metabolic Traits and the Evolution of Host Plant Specialization in Cyanogenic Butterflies

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Received: 16 June 2006 / Revised: 22 August 2006 / Accepted: 13 September 2006 /
Published online: 7 December 2006
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Abstract Larvae of *Heliconius* butterflies (Nymphalidae: Heliconiinae) feed exclusively on cyanogenic leaves of *Passiflora* (passion vine). Most *Heliconius* manufacture cyanogenic glycosides (cyanogens) and some species sequester cyanogens from host plants. We compare ability to sequester simple monoglycoside cyclopentenyl (SMC) cyanogens and manufacture aliphatic cyanogens in 12 *Heliconius* species, including larvae that are specialized (single host species) and generalized (many host species). All butterflies tested higher for cyanide concentrations when reared on plants that larvae can sequester from (SMC plants) than when reared on plants that larvae do not sequester from (non-SMC plants). Specialists in the *sara–sapho* clade sequestered SMC cyanogens from specific host plants at seven times that of *Passiflora* generalists fed the same hosts. In contrast, *sara–sapho* clade species reared on non-SMC plants had significantly lower cyanide concentrations from *de novo* synthesis than generalists fed the same plants. Furthermore, cyanogen analyses indicated that *Heliconius sara* butterflies reared on an SMC host had a greater proportion of sequestered SMC cyanogens (95.0%) than *de novo*-synthesized aliphatic cyanogens (5.0%). Thus, sequestration and *de novo* synthesis are negatively correlated traits. Results suggest that losing the ability to synthesize cyanogens has restricted *sara–sapho* clade species to specific hosts containing SMC cyanogens and explains dietary restriction in this clade.

Keywords Plant–insect interactions · Host plant specialization · Secondary compounds · Cyanogenic glycosides · Sequestration · *De novo* synthesis · Coevolution

We dedicate our paper to the memory of the Honorable Miriam Rothschild, F.R.S., a friend, colleague and supporter of many young scientists. Her enthusiasm and delight in chemical ecology and natural history continued to the last weeks of her life.

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Introduction

Almost 80% of all herbivorous insects are specialized on plants of a single family, genus, or even a single species (Bernays and Graham, 1988; Bernays and Chapman, 1994). Insects may specialize on host plants because of ecological factors such as "enemy-free space" (Gilbert and Singer, 1975; Price et al., 1980; Strong et al., 1984; Bernays, 1989; Gratton and Welter, 1999; Lill et al., 2002; Zangerl et al., 2002; Singer et al., 2004), interspecific competition (Gilbert and Singer, 1975; Strong et al., 1984; Denno et al., 1995), and local host plant abundance (Gilbert and Singer, 1975; Futuyma, 1983; Beccaloni and Symons, 2000; Ballabeni et al., 2003). An alternative explanation is that plant secondary chemistry is the primary driving force for a narrowing of host range in herbivorous insects (Ehrlich and Raven, 1964; Feeny, 1975; Cates, 1980; Cornell and Hawkins, 2003).

Clearly, both chemical and non-chemical factors are important to consider when interpreting patterns of diet breadth in phytophagous insects (Schultz, 1988; Gotthard et al., 2005). Herbivores must deal with plant secondary chemistry, particularly compounds that are potentially harmful to their cellular processes (Karban and Agrawal, 2002). And, although it may be difficult to determine whether plant chemistry is the ultimate basis for host plant specialization, plant chemistry must exert some selection pressure on insect host range (Thompson, 1988).

There are few insect systems for which we have a detailed understanding of the patterns and mechanisms of sequestration and *de novo* synthesis of secondary compounds (Nishida, 2002) and the possible role of these mechanisms in dietary restriction. The *Heliconius–Passiflora* system is of particular value for addressing issues of chemical ecology because of its comparative potential (Gilbert, 1991). Approximately 65 heliconiine species (40 of which are *Heliconius*) and 600 species of *Passiflora* are available for study. Furthermore, a phylogeny is available for both *Passiflora* (Hansen, 2004; MacDougal and Feuillet, 2004) and *Heliconius* (Brower and Egan, 1997; Penz, 1999) (Fig. 1).

Larvae of *Heliconius* butterflies feed exclusively on cyanogenic leaves of *Passiflora* (passion vine). *Passiflora* is a diverse Neotropical genus in which coevolution with *Heliconius* in local populations apparently accounts for morphological innovations in some passion vines (Gilbert, 1971; Benson et al., 1976; Gilbert, 1979; Williams and Gilbert, 1981; Gilbert, 1982; Futuyma, 1983). Cyanogen diversity, particularly the cyclopentenyl cyanogen class (Olafsdottir et al., 1989; Seigler, 1975; Spencer and Seigler, 1984c) (Fig. 2) in passion vines, may be another product of the evolutionary arms race between *Passiflora* and their primary herbivores, species of *Heliconius* butterflies (Spencer, 1988).

Similar to their hosts, species of *Heliconius* are cyanogenic. *De novo* synthesis of aliphatic cyanogenic glycosides (Fig. 2) from amino acid precursors characterizes *Heliconius* species (Nahrstedt and Davis, 1981, 1983). Subsequently, we have shown that sequestration of simple monoglycoside cyclopentenyl (SMC) cyanogens (Fig. 2) characterizes *Heliconius* species that were fed *Passiflora* containing these compounds (SMC plants) (Engler et al., 2000). However, until the present study, no investigation has been made of the relationship between *de novo* synthesis and sequestration of cyanogens in *Heliconius* and the impact of this relationship on patterns of host plant specialization.

In this study, we examine whether larval sequestration of plant allelochemicals has influenced diet breadth in certain species of *Heliconius* butterflies. Specifically, we address the following questions: (1) Given that *Heliconius* sequester cyanogens from SMC plants, does cyanide concentration of plants influence cyanide concentration of *Heliconius* herbivores? (2) As the same *Passiflora* species may be used by specialist and generalist *Heliconius*, do

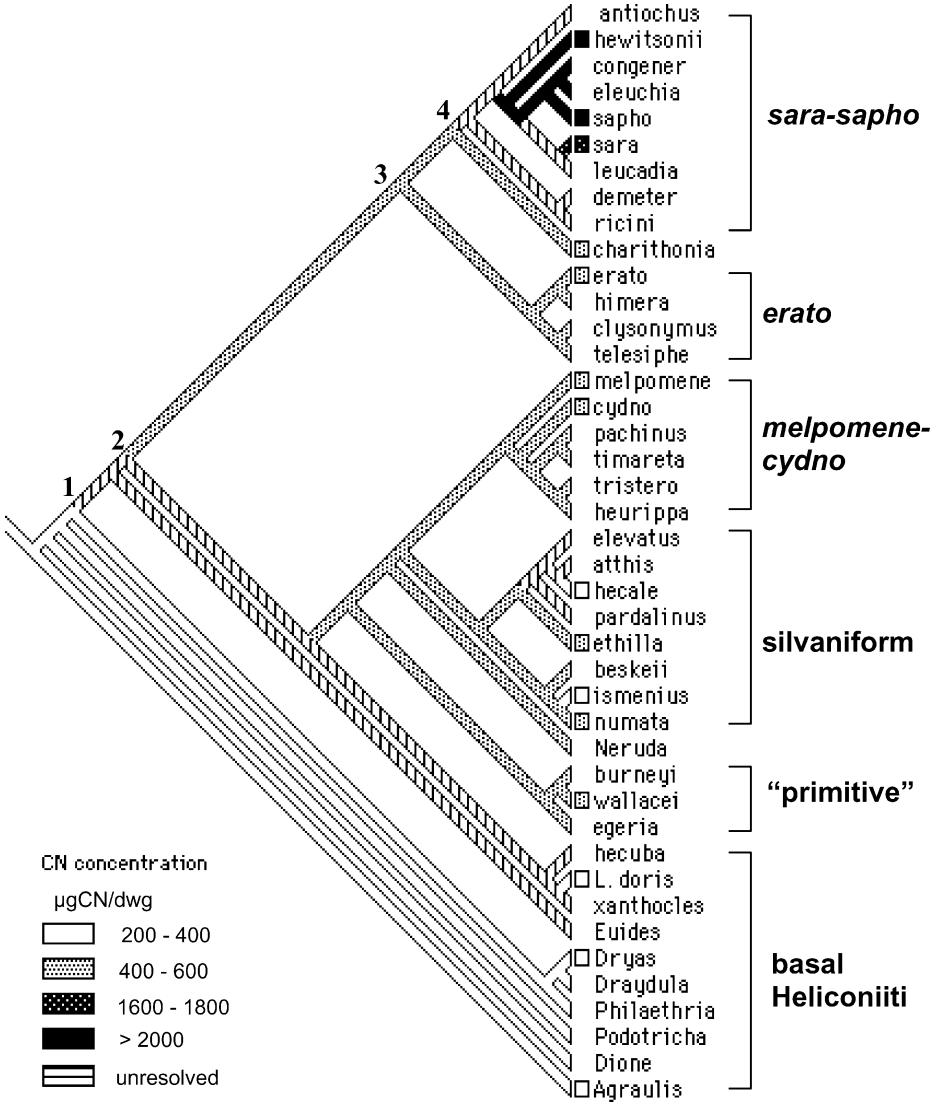


Fig. 1 Annotated phylogeny of the sub-tribe Heliconiiti (Brower and Egan, 1997; Penz, 1999) showing cyanide concentrations of species compared in the study

host specialist *Heliconius* species sequester higher cyanide concentrations from their particular SMC host plants than species with broader host range do? (3) As we know that many, if not most, *Heliconius* and relatives manufacture cyanogens, do those species that sequester lose the ability to carry out *de novo* synthesis?

Methods and Materials

Plants and Insects Our study was conducted at the University of Texas in Austin. Most of the greenhouse populations of heliconiines and Passifloraceae used originated from lowland

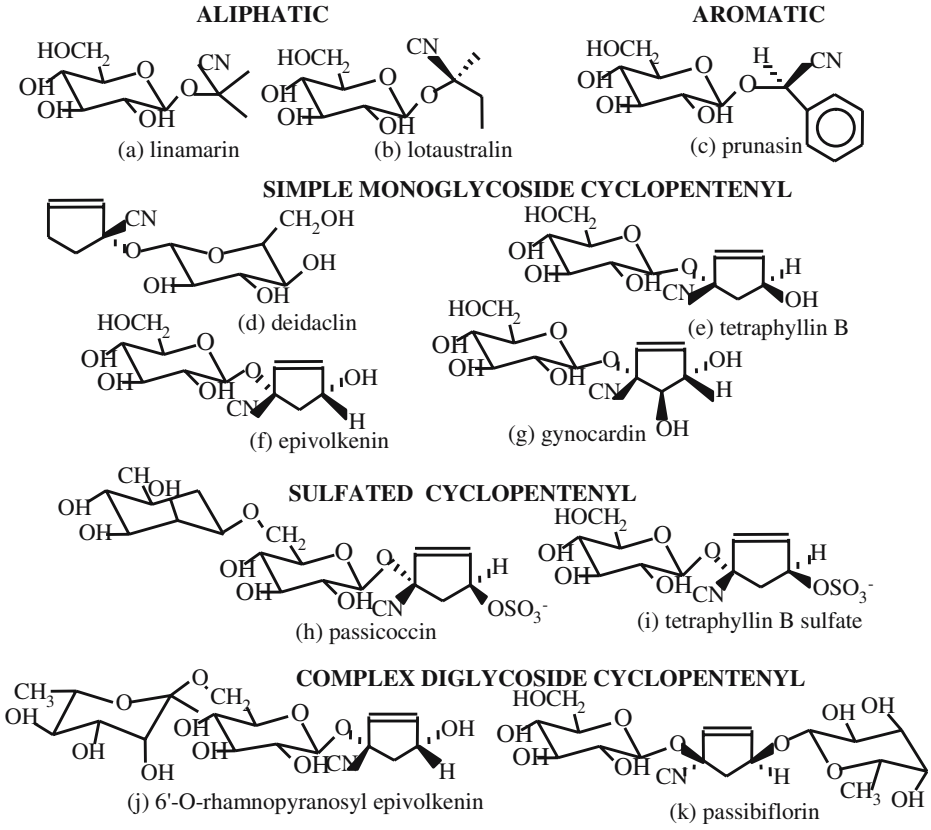


Fig. 2 Structural formulae of the major cyanogenic glycoside (cyanogen) classes found in Passifloraceae (Spencer, 1988; Seigler and Brinker, 1993)

tropical rainforest at the Sirena Field Station in Corcovado National Park, Costa Rica. However, a few species originated from other localities in the neotropics. Most live cultures were established several years before the beginning of our study, but occasional collections of new stocks were made during the study to ensure genetic diversity.

We selected 12 plant species from five subgenera within the Passifloraceae (Killip, 1938). The distribution of cyanogens or cyanogen profile found in the 12 species of Passifloraceae is shown in Table 1 (data derived from Engler, 1998). Host plants from one community (Sirena station in Corcovado Park, Costa Rica) received special focus. In this study, we refer to host plants with simple monoglycoside cyclopentenyl cyanogens that can be sequestered by any *Heliconius* as SMC plants. Host plant taxa without these cyanogens are designated as non-SMC plants. *Passiflora biflora* and *P. vitifolia* were the principal non-SMC host plants utilized in our feeding experiments.

Butterfly Rearing and Feeding Experiments Our study included nine *Heliconius* species from the Pacific slopes and lowlands of Costa Rica. Three *Heliconius* species originated from South America, including *H. ethilla* (South Brazil), *H. numata* (Amazonian Ecuador), and *H. wallacei* (Amazonian Ecuador). We included some close relatives to *Heliconius*, including *Agraulis vanillae* (Austin, TX, USA), *Dryas julia* (Costa Rica), and *Laparus*

Table 1 Cyanogen profiles showing distribution of cyanogen classes among species of passifloraceae used in the study

				Cyanogen Classes				
				Aliphatic	Aromatic	Cyclopenentenoid		
						Simple [b]	Sulfated	Sulfated/ Complex
						Monoglycoside		Diglycoside
Passifloraceae	Plant Subgenus	Accession Number	Plant [a] Origin					
Tetrastylis ovalis (ova)	*Deidamioides*	8071	1	+		+		
Passiflora pittieri (pit)	*Astrophea*	8048	2			+	+	
P. auriculata (aur)	*Decaloba*	8028	2			+		
P. coriacea (cor)	*Decaloba*	9217	2	+		+		+
P. biflora (bif)	*Decaloba*	6001	3					+
P. talamancensis (tal)	*Decaloba*	8067	2					+
P. lobata (lob)	*Decaloba*	9243	2	+				
P. menispermifolia (men)	*Passiflora*	9224	2	+				
P. oerstedii (oer)	*Passiflora*	7005	3			+	+	
P. edulis (edu)	*Passiflora*	9058	4		+		+	
P. quadrangularis (qua)	*Passiflora*	8054	2				+	
P. vitifolia (vit)	*Passiflora*	8070	2					+

+: presence of cyanogen class.

[a] Origin of seed or cutting: (1) Espirito Santo, Brazil; (2) P.N. Corcovado, Costa Rica; (3) Nr. Puerto Viejo, Costa Rica; (4) Pt. Alegre, Brazil.

[b] Host plants with simple monoglycoside cyclopentenyl cyanogens (SMC plants) are shaded.

doris (Costa Rica), to expand our representation of the subtribe *Heliconiiti* (Brower and Egan, 1997; Penz, 1999).

Heliconius species generally utilize a small subset of *Passiflora* available in microhabitats and preferred by each species for reproductive activity (Gilbert, 1991). To appropriately describe the patterns of diet in larval *Heliconius*, we use the terms specialist (single host species), oligophagous (few host species), and generalist (many host species) to indicate the relative degree of restriction of various species of *Heliconius* to species of *Passiflora* available in a local site. Patterns of larval host plant use and diet breadth among *Heliconius* species (Benson et al. 1976; Brown, 1981; Smiley 1978, 1985) used in the study are shown in Table 2.

Butterfly cultures were maintained as free flying, continuously breeding populations under controlled, semi-natural environmental conditions in (5×8 m) glass greenhouses on the roof of Patterson Laboratories (Austin, TX, USA). Host plants have been grown in these facilities continuously, allowing long-term persistence of stock populations. Healthy cultures depend upon the availability of suitable *Passiflora* larval host plants, and appropriate adult nectar and pollen resources (*Psiguria*, *Gurania*, *Psychotria*, and *Lantana* species) (Gilbert, 1972; O'Brien et al., 2003). Adults were also provided two supplements to floral resources. First, a synthetic diet based on the amino acid content of *Psiguria* pollen, plus

Table 2 Patterns of larval host plant use among groups of *Heliconius* and specialists used in the study

Heliconius group	Larval diet breadth	*Passiflora* host plant(s)
sara–sapho	Specialist	Primarily subgenus *Astrophea*
H. sapho		*P. pittieri*
H. hewitsoni		*P. pittieri*
H. sara		*P. auriculata*
charithonia	Oligophagous	Subgenus *Decaloba*
erato	Oligophagous	Subgenus *Decaloba*
melpomene–cydno	Generalist[a]	Wide range of *Passiflora* species
H. melpomene		*P. oerstedii*
Silvaniform	Generalist	Wide range of *Passiflora* species
"Primitive"	Specialist	Subgenus *Passiflora*
H. wallacei		*P. vitifolia*

Specialists are shaded.

Specialist: single host species; oligophagous: few host species; generalist: many host species

[a] *H. melpomene*, although not a larval specialist, is an oviposition specialist on *P. oerstedii*.

25% sucrose solution, was sprayed onto yellow and orange "Tuffy" plastic scrubbing pads and allowed to dry. Second, 25% sucrose solution was continuously presented in styrofoam cups that contained Tuffy pads for an attractive signal and landing platform. All of these solutions were changed once per week.

For each experimental assay, larvae were reared from egg to adult on plants within an environmental chamber at 25°C and 80% RH. The average life cycle of *Heliconius*, from egg to adult, is approximately 25 d. Newly hatched larvae were fed young leaves and tendrils in individual two-ounce (50 ml) condiment cups. Third instar larvae were moved to ventilated, 1-gallon plastic aquaria or 0.3×0.3×0.3 m screen cages. Older larvae were fed fresh leaves on stem cuttings whose cut ends were kept submerged in distilled water in 25-ml bottles. Pupae were hung in sealed, humidified 1-gallon plastic aquaria. Larval rearing containers were cleaned on a daily basis. After butterflies emerged, teneral butterflies were stored in double-bagged plastic containers and prepared for cyanogen analyses.

It is important to note that it was virtually impossible to rear *Heliconius* species of the *sara–sapho* clade on more than a couple of non-SMC plants because of their high level of larval host specificity. To successfully rear specialized larvae on non-host plants, we moved second instar larvae from their normal host to the new host. This sufficed for our cyanogen analyses, but larvae had lower viability.

Quantification of Cyanogens in Plants and Butterflies Using Colorimetric Analysis We quantified cyanogen levels in SMC and non-SMC plants and in butterflies reared on SMC and non-SMC plants using the colorimetric method of Lambert et al. (1975), as modified by Brinker and Seigler (1989) and Schappert and Shore (1995). By this method, we estimate cyanogen levels in plants and insects by the amount of hydrogen cyanide (HCN) released from crushed samples.

For *Passiflora* assays, six 0.6-cm leaf disks were sampled from living plants of each species. Three age categories of leaves, as indicated by length of midvein (cm), were sampled from each plant to control for developmental variation in cyanide concentration. In the cyanophoric *P. oerstedii*, it was necessary to add exogenous β-glucosidase enzyme, prepared from *Gynocardia odorata*, to drive cyanide out of the plant tissue for quantification. This is because *P. oerstedii*

contains an SMC cyanogen but lacks β-glucosidase enzymes (Engler-Chaouat, unpublished data). For *Heliconius* assays, teneral adult butterflies were quantified individually. It was not necessary to add exogenous β-glucosidase enzyme to any butterfly samples as determined from a previous pilot experiment.

Each plant or butterfly sample was ground with a plastic tissue homogenizer in a 1.5-ml microcentrifuge tube. Immediately after homogenization of samples, the tubes were placed uncapped in individual 20-ml glass scintillation vials, with 0.5 ml 1.0 M NaOH covering the bottom surface of each vial. Vials were sealed tightly and incubated at 37°C for 24 h. Under these conditions, β-glucosidase enzymes hydrolyze cyanogens and release HCN. In solution, CN^- ions are trapped in the NaOH as NaCN. Previous analyses have shown that all detectable HCN is driven out of samples in 24 h (Brinker and Seigler, 1989; Schappert and Shore, 1995), and we confirmed this finding.

After the incubation period, a 0.1-ml aliquot of each 1.0 M NaOH/NaCN sample was placed in a 20-ml test tube and diluted with 0.9-ml of double distilled water. The following reagents were added to each tube: 0.5 ml 1.0 M acetic acid, 5.0 ml succinimide/N-chloro-succinimide reagent, and 1.0 ml barbituric acid/pyridine (Lambert et al., 1975; Brinker and Seigler, 1989). The tubes were vortexed at room temperature and, after a 10-min color reaction period, a 1.0-ml aliquot of each sample was measured at 580 nm in a Shimadzu™ spectrophotometer.

Optical density (OD) values were used to estimate cyanide concentrations based upon a calibration curve performed with known NaCN concentrations. A strong linear relationship exists between OD and μg HCN liberated using this method (R^2=0.99, P<0.001). Crushed samples were dried to constant mass for 4 to 6 d in a drying oven at 67°C. Dry mass of samples was used, in conjunction with the calibration curve, to calculate micrograms of HCN liberated per gram dry weight of tissue (μg CN/dwg) (Tables 3, and 4).

Quantification of Cyanogens in Butterflies Using Chromatographic Analysis We quantified the proportion of aliphatic and SMC cyanogens in specialist, *Heliconius sara*, using paper chromatography (PC) and high performance liquid chromatography (HPLC) following published methods (Jaroszewski et al., 1987; Seigler and Brinker, 1993; Spencer, 1988). First, cyanogens were extracted from 14 teneral female (total dry mass=0.55 g), and 21 teneral male (total dry mass=0.50 g) *H. sara* that were reared on their specific SMC host *P. auriculata*. To extract cyanogens, butterflies were ground in cold 80% methanol, filtered,

Table 3 Mean cyanide concentration and standard error in host plants used in the study

Passifloraceae	CN (μgCN/dwg)±SE	*N*
Tetrastylis ovalis	1,986±219	7
Passiflora pittieri	4,474±950	10
P. auriculata	474±74	32
P. coriacea	6,020±1,233	8
P. biflora	1,542±178	25
P. talamancensis	12,418±1,330	7
P. lobata	115±53	8
P. menispermifolia	3460±471	7
P. oerstedii[a]	1171±126	18
P. edulis	2959±446	7
P. quadrangularis	1931±230	22
P. vitifolia	532±28	20

N number of plants

[a] Cyanophoric *P. oerstedii* required the addition of exogenous *Gynocardia odorata* enzyme for cyanide release.

Table 4 Mean cyanide concentration[a] and standard error of species of heliconiine butterflies reared on passifloraceae hosts

	Passifloraceae									
Heliconius	ova	pit	aur	bif	lob	men	oer	qua	amb	vit
sapho		2423 ± 586 (9)		9 ± 3 (5)						
hewitsoni		2371 ± 103 (13)								
sara	613 ± 46 (10)	496 ± 73 (4)	1695 ± 80 (46)	64 ± 19 (29)						
charithonia		664 ± 68 (5)	583 ± 70 (7)	574 ± 45 (35)	473 ± 47 (3)					630 ± 57 (4)
erato			588 ± 216 (2)	455 ± 113 (4)						
melpomene		719 ± 72 (4)	223 (1)	236 ± 101 (4)		375 ± 51 (6)	470 ± 39 (18)			335 ± 21 (5)
cydno		590 (1)	382 (1)	518 ± 32 (48)		451 ± 5 (2)	587 ± 26 (5)			562 ± 52 (9)
hecale		355 ± 119 (4)	419 ± 25 (15)	217 ± 19 (50)		378 ± 38 (2)	475 ± 53 (6)			385 ± 29 (12)
ethilla		465 ± 73 (3)	677 ± 113 (6)	495 ± 59 (15)		613 (1)	789 (1)			550 ± 82 (6)
ismenius		650± 155 (4)	583 ± 34 (8)	245 ± 35 (22)			652 ± 60 (3)	309 ± 23 (23)		179 (1)
numata		509 ± 55 (17)		399 ± 38 (28)			501 ± 120 (2)			499 ± 47 (16)
wallacei										561± 40 (15)
Relatives										
D. julia			372 ± 37 (4)	352 ± 46 (5)						
A. vanillae				320 ± 67 (5)						
L. doris[b]									357 ± 29 (3)	

Number of butterflies is indicated in parentheses. Host plants with simple monoglycoside cyclopentenyl cyanogens (SMC plants) are shaded.

[a] Cyanide concentration of samples was measured as micrograms of HCN liberated per gram dry weight of tissue (μgCN/dwg).

[b] *L. doris* samples were the only butterflies reared in the field.

dried, and partitioned exhaustively between $CHCl_3$ and H_2O several times. After cyanogen extraction, the aqueous layer was run on Whatmann 3-mm chromatography paper with 6:3:1 (Isoprop: *n*-but: H_2O) as an eluent. The aliphatic (R_f=0.50) and SMC (R_f=0.37) cyanogen bands were cut out and desorbed separately in H_2O from a total of 30 PCs. Finally, cyanogen products were concentrated under vacuum and prepared for further purification.

After paper chromatography, the partially purified aliphatic and SMC cyanogen isolations were separated using a SSI HPLC [C-18 Beckman column (5 μm, 2 mm×25 cm], 5.0% methanol in water, flow rate 0.18 ml/min, refractive index detector). Under these conditions,

all cyanogens, except enantiomers, elute as separate peaks. Relative retention times of eluted cyanogens were calibrated with reference standards including aliphatic cyanogen linamarin (8.9 min), and SMC cyanogens, epivolkenin (7.4 min), and gynocardin (5.3 min). The proportion of aliphatic and SMC cyanogen compounds in *H. sara* was calculated based on the percent area of chromatogram peaks.

Chemical Correlates of Host Plant Use All statistical analyses were carried out using Statview. Quantitative differences in *Passiflora* host plants and in butterfly species using these hosts were analyzed using regression analysis and analysis of variance (ANOVA) (Sokal and Rohlf, 1981).

Our first analysis addressed question 1: Do cyanide concentrations of SMC and non-SMC plants differ in influencing cyanide concentrations of *Heliconius* herbivores? We performed two regression analyses to test whether cyanide concentration in host plants is related to cyanide concentrations in *Heliconius* butterflies. Host plants utilized at the source locality of each *Heliconius* species were used in each analysis. The first regression related the mean cyanide concentrations (μgCN/dwg) of butterflies that were fed SMC host plants to the mean cyanide concentration (μg CN/dwg) of the SMC host plants. The second regressed mean cyanide concentrations of butterflies that were fed non-SMC host plants (*P. biflora* and *P. vitifolia*) against mean cyanide concentration of these non-SMC host plants.

Our second analysis addressed question 2: Do host specialist *Heliconius* species sequester higher cyanide concentrations from their particular SMC host plants than species with broader host range do? Using ANOVA, we compared mean cyanide concentrations of butterflies reared on SMC host plants (*P. auriculata*, *P. pittieri*, and *P. oerstedii*) with mean cyanide concentrations of the same butterflies reared on a non-SMC plant (*P. biflora*). Two-way ANOVAs were performed with butterfly and host plant species as independent factors and mean cyanide concentration of butterflies as the dependent variable. These comparisons also allowed us to address question 3: Do those *Heliconius* species that sequester lose the ability to carry out *de novo* synthesis?

Phylogenetic Associations of Host Specialization, Sequestration, and Cyanogenesis Given that species can share traits due to common ancestry, species data points may not be independent in comparative studies. Therefore, to account for phylogeny, we reanalyzed our data using an independent contrasts program [comparative analysis by independent contrasts (CAIC); Felsenstein, 1985; Harvey and Pagel, 1991; Garland et al., 1993; Purvis and Rambaut, 1995] to control for the independence of species (Irschick et al., 2005). We compared mean cyanide concentrations of 12 *Heliconius* species and two close relatives reared on SMC and non-SMC plants using CAIC and regression analysis.

Three CAIC analyses were employed to control for host plant differences. In the first analysis, we computed contrasts for mean cyanide concentrations of butterflies reared on a SMC plant. The SMC plant was either *P. auriculata* or *P. pittieri*, in most cases. In the second analysis, we computed contrasts for mean cyanide concentrations of butterflies reared on a natural host plant at the source locality. In the third analysis, we computed contrasts for mean cyanide concentrations of butterflies reared on a non-SMC plant, either *P. biflora or P. vitifolia*.

CAIC reduced our data to four sequential nodes of the *Heliconiiti* tree, beginning at the base (basal *Heliconiiti*) and moving out to the tips (*sara–sapho*) of the tree (see Fig. 1). Topological data for the *Heliconiiti* phylogeny, obtained from Brower and Egan (1997) and Penz (1999), were coded for independent contrasts analysis. Relative branch lengths in the phylogeny were coded assuming that the ages of taxa are proportional to the number of

species (Grafen, 1989; Purvis and Rambaut, 1995) We repeated each analysis assuming equal branch lengths as a control.

Standardized linear contrasts were computed at each node of the tree using a "Crunch" algorithm for continuous characters. Contrasts began at the tips of the tree and worked back to the root of the tree. "Brunch" is preferred for dichotomous data, but it produced too few contrasts for statistical analysis. Purvis and Rambaut (1995) report that Crunch is acceptable for most analyses.

After each CAIC analysis, the three sets of contrasts were analyzed using three regression analyses. In all regression analyses, sequestration from the primary host plant was the categorical, independent variable. In other words, species were considered "sequesterers" if an SMC plant was a primary host plant at source localities. Cyanide concentration was the dependent variable. (Purvis and Rambaut, 1995; Sokal and Rohlf, 1981). Only nonzero contrasts of the independent variable and dependent variable were plotted, as contrasts of zero indicated no difference between the taxa compared. Positive contrasts in the dependent variable indicate variation between taxa driven in the same direction as the independent variable. Negative contrasts indicate variation between taxa driven in the opposite direction to the independent variable. All regressions were forced through the origin as required by CAIC.

Results

Chemical Correlates of Host Plant Use

1. Does cyanide concentration of plants influence cyanide concentration of *Heliconius* herbivores? We found no relationship between the mean cyanide concentration of *Passiflora* host and the mean cyanide concentration of butterfly herbivores. The cyanide concentration of butterflies reared on non-SMC plants (*P. biflora* and *P. vitifolia*) was not correlated with the cyanide concentration found in these host plants (r^2=0.09; P=0.1758). Similarly, the cyanide concentration of butterflies reared on SMC plants (*P. auriculata*, *P. pittieri*, and *P. oerstedii*) was not significantly related to the cyanide concentration of these SMC plants (r^2=0.071; P=0.2689). However, butterflies reared on SMC plants (*P. auriculata*, *P. pittieri*, and *P. oerstedii*) generally had significantly higher cyanide concentrations than the same butterflies reared on non-SMC plant, *P. biflora* (Fig. 3), indicating that sequestration of SMC cyanogens increases the mean cyanide concentration in the butterfly herbivore.
2. Do host specialist *Heliconius* species sequester higher cyanide concentrations from their particular SMC host plants than species with broader host range do? Oviposition and larval specialist species from the *sara–sapho* clade, *H. hewitsoni*, *H. sapho*, and *H. sara*, had significantly higher cyanide concentrations, as high as seven times that of generalist species reared on their specific SMC host plants, *P. pittieri*, *P. pittieri*, and *P. auriculata*, respectively (Table 4, Fig. 3). In contrast to the situation observed in the *sara–sapho* clade, oviposition specialist *H. melpomene* did not have higher cyanide concentrations than other butterflies reared on its SMC host, *P. oerstedii*. These data also show that although *P. pittieri* is a SMC plant, *H. sara* does not contain significantly higher cyanide concentrations than generalist *Heliconius* when reared on this plant. This indicates a significant degree of specialization by *H. sara* within the set of different SMC host species.

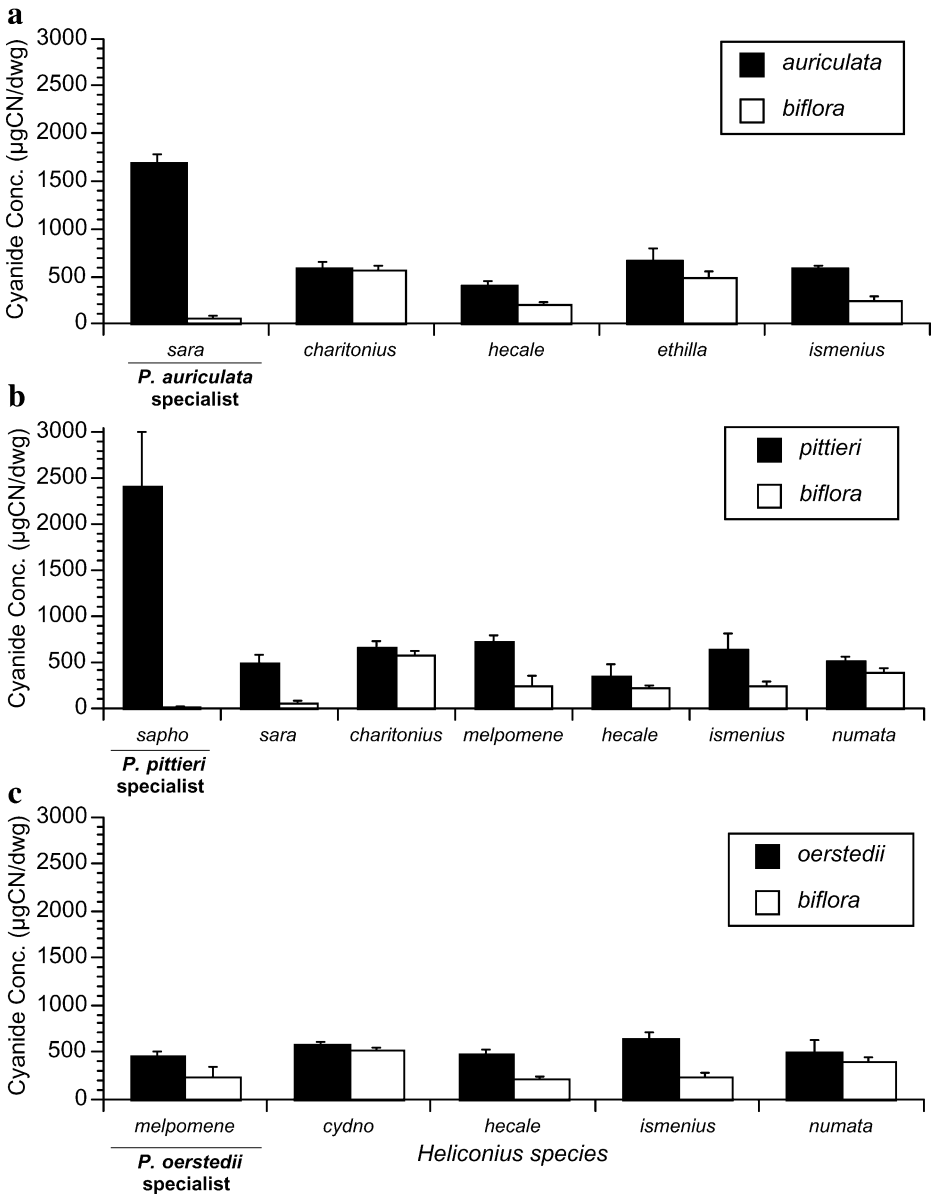


Fig. 3 Mean cyanide concentrations of specialist and generalist species of *Heliconius* reared on SMC host plants, *P. auriculata*, *P. pittieri*, *P. oerstedii*, and the non-SMC host *P. biflora*. (A) Butterflies: $F_{4,\ 222}$= 26.948, P<0.001; plants: $F_{1,\ 222}$=81.689, P<0.001; butterflies × plants: $F_{4,\ 222}$=63.406, P<0.001; (B) butterflies: $F_{6,\ 203}$=5.421, P<0.001; plants: $F_{1,\ 203}$=46.767, P<0.001; butterflies × plants: $F_{6,\ 203}$=8.702, P<0.001; (C) butterflies: $F_{4,\ 176}$=3.998, P=0.0040; plants: $F_{1,\ 176}$=22.221, P<0.001; butterflies × plants: $F_{4,\ 176}$=1.807, P=0.1295

3. Do those *Heliconius* species that sequester lose the ability to carry out *de novo* synthesis? Species in the *sara–sapho* clade, *H. sapho* and *H. sara*, had significantly lower cyanide concentrations, by a mean factor of 10 times that of generalist species, when they were reared on a non-SMC host (Table 4, Fig. 3). Chromatographic analyses also showed that *H. sara* butterflies fed their specific SMC host, *P. auriculata*, had a greater proportion of sequestered SMC cyanogens (95.0%) than *de novo* synthesized aliphatic cyanogens (5.0%). This demonstrates a negative relationship between the ability of butterflies to sequester SMC cyanogens and to *de novo* synthesize aliphatic cyanogens.

Phylogenetic Associations of Host Specialization, Sequestration, and Cyanogenesis The relationship between host specialization, sequestration, and cyanogenesis depends heavily on whether or not host plants produce simple monoglycoside cyclopentenyl (SMC) cyanogenic compounds. Cyanogenesis in the butterfly can be attributed to *de novo* synthesis and sequestration when host plants produce SMC cyanogens, whereas cyanogenesis in the butterfly can be attributed to only *de novo* synthesis when host plants contain non-SMC cyanogens.

We found a highly significant, positive relationship between sequestration from the primary host plant and the cyanide concentration of the butterfly among butterflies reared on SMC plants (Fig. 4) (r^2=0.987, P<0.001). Similarly, we found a highly significant, positive relationship between sequestration from the primary host plant and the cyanide concentration of the butterfly in comparing butterflies reared on natural host plants from their source locality (Fig. 4) (r^2=0.988, P<0.001). The statistical significance of this result is driven by the fact that the primary host plants of *sara–sapho* clade species are SMC plants. By contrast, in comparing species reared on a non-SMC plant, there was a highly significant, negative relationship between sequestration from the primary host plant and the cyanide concentration of the butterfly (Fig. 4) (r^2=0.916, P=0.0016). Thus, sequestration of SMC cyanogens and *de novo* synthesis of aliphatic cyanogens are negatively correlated metabolic traits in passion vine butterflies.

Discussion

This research adds significantly to the knowledge of sequestration and *de novo* synthesis of cyanogenic glycosides in heliconiine butterflies. More importantly, our results have broad implications for understanding the evolution of host plant specialization in phytophagous insects and factors, such as sequestration, contributing to such specialization. Although it has been suggested that monophagy is associated with effective sequestration of secondary chemicals in the Lepidoptera (Duffey, 1980; Bowers, 1992; Dyer, 1995; Nishida, 2002, ours is the first interspecific comparative study to demonstrate a correlation between host plant specialization and sequestration.

Ecological factors may drive the evolution of host specificity in herbivorous insects (Zangerl et al., 2002; Ballabeni et al., 2003), including species of *Heliconius* (Smiley, 1985; Gilbert, 1991). In the present study, however, we highlight an instance in which the secondary chemistry of host plants drives the evolution of specificity among some, but not all, of the insect herbivores that use them as larval hosts. Specifically, our study suggests that physiological mechanisms involved in sequestration of SMC cyanogens and consequent loss of *de novo* synthesis of cyanogens might be a factor promoting and enforcing dietary restriction to SMC host plants in a derived clade of *Heliconius* butterflies.

Studies of chemical factors influencing diet breadth have led to the "trade-off" or "metabolic load" hypothesis (Whittaker and Feeny, 1971; Rausher, 1988; Jaenike, 1990). Under this hypothesis, as specialist insects adapt their physiology (i.e., metabolic enzymes) to sequester or counter specific host plant allelochemicals (Duffey, 1980; Bowers, 1990), they lose their ability to deal with non-host plant toxins (Cohen, 1985; Bowers and Collinge, 1992; Rowell-Rahuer and Pasteels, 1992; Adler et al., 1994; Joshi and Thompson, 1995; Camara, 1997). However, few studies have provided support for this hypothesis (Hare and Kennedy, 1986; Futuyma and Phillipi, 1987; Bernays, 1988; Bowers, 1988; Kearsley and Whitham, 1992;

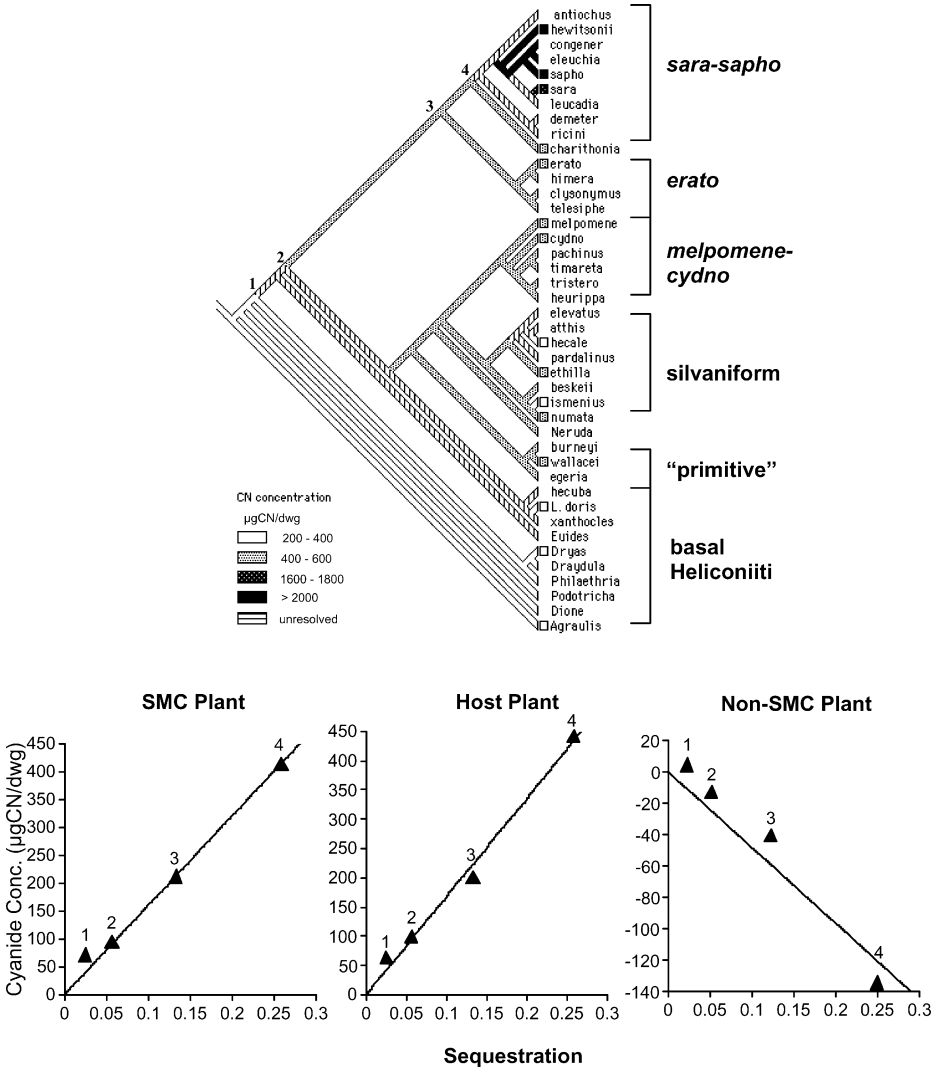


Fig. 4 Regressions of phylogenetic contrasts of sequestration and cyanide concentration of *Heliconius* reared on either an SMC plant (r^2=0.987, P<0.001), natural host plant (r^2=0.988, P<0.001), or non-SMC plant (r^2=0.916; P=0.0016). The numbers 1–4 represent CAIC analysis contrasts at four nodes of the *Heliconiiti* tree

Fry, 1996; Camara, 1997; but see Cornell and Hawkins, 2003) and tradeoffs may be difficult to detect (Fry, 2003).

We show in our study of *Heliconius* that several species of a derived clade (*sara–sapho*), characterized by the highest degree of host restriction as adults and larvae, sequester SMC cyanogens from specific host plants at significantly higher concentrations than that of generalists fed the same hosts. In contrast, these same species reared on non-SMC plants manufactured significantly lower cyanide concentrations than generalists fed the same plants. Thus, we demonstrate that the ability of butterflies to sequester SMC cyanogens and to *de novo*-synthesize aliphatic cyanogens are negatively correlated traits in passion vine butterflies. This result constitutes evidence for an evolutionary "tradeoff" between sequestering plant-derived cyanogenic compounds *versus* manufacturing cyanogenic compounds *de novo*.

One possibility to consider is that *de novo* synthesis is resumed in adult *Heliconius*, even when it is absent in the larval stage. If adult *Heliconius* synthesize cyanogens from amino acid precursors obtained from pollen feeding as has been suggested (Gilbert, 1991), it would follow that any lack of cyanogen synthesis capability by larvae could be compensated by adults of the *sara–sapho* clade. However, in a preliminary screen, we found no evidence of aliphatic cyanogen synthesis in older adults of *H. sapho* (Engler, unpublished data). Therefore, we suggest that the virtual loss of *de novo* synthesis of cyanogens in the *sara–sapho* clade, and a subsequent "chemical dependency" (Brown et al., 1991) on host plants for acquiring and maintaining high cyanide levels, might explain why the *sara–sapho* species are restricted to SMC host plants.

Several lines of evidence suggest that the physiological mechanism(s) involved in such "high level" sequestration, as shown by *sara–sapho* clade, is not simply because of the presence of SMC cyanogens in host plants nor of their being a specialist. First, although all tested butterflies reared on SMC plants had higher cyanide concentrations than the same butterflies reared on non-SMC plants, only members of the *sara–sapho* clade had extremely high cyanide levels when reared on their SMC hosts. Second, Central American *H. sara* used in the study does not store significantly higher cyanide levels from SMC plant *P. pittieri* than other *Heliconius* species, although many South American populations of *H. sara* specialize on *pittieri*-like species of Astrophea. Third, H. *melpomene*, an oviposition specialist, does not store higher levels of cyanide than other *Heliconius* species reared on its specific SMC host plant in the Atlantic forests of Costa Rica, *P. oerstedii*.

Furthermore, adult and larval specialist *H. wallacei* does not have significantly higher concentrations than generalists reared on its non-SMC host plant, *P. vitifolia*. Thus, the extent of physiological specialization by both adults and larvae sets the *sara–sapho* clade apart from all other species of the genus *Heliconius* studied so far, including those that are strict specialists on non-SMC plants, and those, on the other hand, that are able to incorporate SMC plants into a broader diet.

Our work raises a puzzling question regarding why a group of insects that can synthesize cyanogens *de novo* would evolve cyanogen sequestration in the first place. It has been suggested that the ability of *Heliconius* and related genera to manufacture and store aliphatic cyanogens (Nahrstedt and Davis, 1981, 1983) provided protoheliconiines with a ready-made physiological mechanism for colonizing cyanogenic *Passiflora*. (Rothschild, 1971; Nahrstedt and Davis, 1985; Raubenheimer, 1989). However, physiological mechanisms involved in cyanogen biosynthesis may be very different from those mechanisms used for cyanogen resistance. As SMC cyanogens are common in ancestral *Passiflora* plants (Spencer, 1988), we suggest that sequestration of SMC cyanogens may have also enabled protoheliconiines to colonize basal Passifloracea such as the woody vines and trees of

subgenus, *Astrophea*. Raubenheimer (1989) also suggests that related *Acraea* may have diversified on cyanogenic plants because of its capability to sequester a SMC cyanogen, gynocardin. With the appearance of the derived *sara–sapho* group, *Heliconius* apparently reinvaded and radiated across ancestral woody passion vines (Benson et al., 1976), and sequestration of host cyanogens might be considered the innovation fueling this adaptive radiation (Gilbert, 2003).

The *sara–sapho* clade represents a recent adaptive zone within the genus *Heliconius*. But, what is the advantage of this specialization over more generalized species that can eat the same SMC plants? Are the larvae more toxic and thus able to group up and deal with the tough leaves of Astrophea? Are the eggs better protected from predators, especially as egg clusters laid by *sara–sapho* females? Are young adults more toxic without having to collect pollen first for cyanogens synthesis?

Ithomiine butterflies as a group do not sequester, and they are very edible until they start collecting pyrrolizidine alkaloids. But like our case, some are now known to sequester from larval hosts (Freitas et al., 1996).

We show that new adults of some members of the *sara–sapho* clade are more cyanogenic than the others and they sequester a more toxic class of cyanogens (Spencer, 1988), thus increasing distastefulness quantitatively as well as qualitatively. That could provide an important degree of protection and predicts these will be the models in intra-generic mimicry. In fact, one race of *H. cydno* has become polymorphic, mimicking two members of the *sara–sapho* clade in Ecuador, *H. sapho* and *H. eluchia*, and generally the polymorphic mimic is the more edible one (Kapan, 2001).

The genus *Heliconius* is already known for species that are apparently specialized on *Passiflora* for ecological reasons (Gilbert, 1991). This study reveals that some *Heliconius* species are monophagous because of physiological mechanisms involved in sequestration. Thus, we have added an important new twist to our knowledge of ecological and evolutionary interaction of *Heliconius* and *Passiflora*, increasing the applicability of this diverse system to the comparative analysis of plant–herbivore chemical interaction and coevolution.

Acknowledgments We thank K. Spencer for the training, equipment, and chemicals that made the work possible, and P. Schappert for the technical advice. K. Spencer, M. Cardoso, P. Schappert, C. Penz, E. Deinert, P. Chai, and R. Dudley contributed useful discussion and criticisms. We thank S. Bramblett for computer graphics, and undergraduates A. Gruetzmacher, R. Saberi, and G. Parikih for assistance in lab and insectary. We thank C. Penz and D. Kapan for providing Ecuadorian material via permits from INEFA and W. W. Benson for Brazilian material via permit with IBAMA. We thank the officials of MINENEM for permits to collect and export butterflies from Costa Rica, and to the staff of Corcovado National Park. Importation of butterflies was enabled by permits from USDA-APHIS. Greenhouse facilities were developed through NSF grants DEB-79060332 and BRS-8315399 with the support of U.T. Austin. Funding for this project was provided by the School for Biological Sciences and the Office of the VP for Research, U.T. Austin.

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J Chem Ecol (2007) 33:43–57
DOI 10.1007/s10886-006-9209-6

Changes in Volatile Production During the Course of Fungal Mycelial Interactions Between *Hypholoma fasciculare* and *Resinicium bicolor*

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Received: 28 March 2006 / Revised: 20 September 2006 / Accepted: 25 September 2006 /
Published online: 5 December 2006
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Abstract The mycelia of two wood decay basidiomycete fungi were grown opposing each other across a 1-μm pore membrane supported on the surface of malt broth, contained within a sealable reaction vessel. Production of volatiles during the time course of interaction was followed by collecting head space samples by solid phase microextraction (100 μm polydimethylsiloxane fiber) on five occasions over 25 d following coinoculation of the fungi: 1, 3 (i.e., immediately prior to mycelial contact), 9 (1–2 d after initiation of pigment production by *Resinicium bicolor*), 17, and 25 d. Ten volatiles were produced during interactions that were not detected in single species controls. In general, most (18) fungal volatiles were sesquiterpenes eluted between 12.5 and 21 min, with a further two eluted at 29.1 and 33.9 min; a benzoic acid methyl ester, a benzyl alcohol, and a quinolinium type compound with a distinctive fragmentation pattern at *m/z* 203, 204, 206, and 207 were also identified; three volatiles with *m/z* maxima of 163, 159, and 206–208, respectively, remained unidentified. The results are discussed in relation to possible ecological roles of volatiles.

Keywords Sesquiterpene hydrocarbons · Benzoic acid methyl ester · Benzyl alcohol · Insect attraction · Fungal volatiles · GC/MS

Introduction

During growth through organic resources and soil, basidiomycete mycelia frequently encounter mycelia of other fungi, both of the same and of different species (Rayner and Boddy, 1988; Boddy, 2000). When basidiomycete mycelia meet, recognition as "non-self" (both inter- and intraspecifically) results in antagonistic responses (unless the mycelia are mating compatible) accompanied by changes in morphology in the interaction zone (and often elsewhere) and in production of extracellular enzymes, volatile and diffusible secondary metabolites, often visually manifested as bright pigments in mycelium and

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culture substratum (Rayner and Boddy, 1988; White and Boddy, 1992; Griffith et al., 1994a,b,c; Score et al., 1997). Mycelial growth is often stopped, reduced, or stimulated, and such responses are often induced prior to contact (Schoeman et al., 1996; Wheatley et al., 1997; Savoie, 2001; Humphris et al., 2002; Wald et al., 2004a,b), but also following contact, and in the case of intraspecific interactions, following hyphal fusion (Rayner and Boddy, 1988; Boddy, 2000). Responses occurring prior to contact are likely to be induced by diffusible and/or volatile organic compounds (VOCs) depending on the combination of interacting species (Rayner et al., 1994; Heilmann-Clausen and Boddy, 2005). In this paper, we concentrate on VOCs.

VOCs have been detected from fungal fruit bodies (Fäldt et al., 1999), fungal-spoiled food (de Lacy Costello et al., 2001; Demyttenaere et al., 2004), and fungi within buildings (Korpi et al., 1999; Ewen et al., 2004). VOCs produced by basidiomycetes and ascomycetes include alcohols, terpenes, aldehydes, ketones, sesquiterpenes, and aromatic compounds (Sunesson et al., 1995; Wheatley et al., 1997; Korpi et al., 1999; Rösecke and König, 2000; Rösecke et al., 2000; Abraham, 2001; Wheatley, 2002). Many sesquiterpenes from plants (e.g., Cakir et al., 2005; Cheng et al., 2005; Wang et al., 2005) and fungi (e.g., Florianowicz, 2000; Abraham, 2001; Roy et al., 2003) appear to have antifungal properties, and VOCs produced by some fungi have been considered as potential biocontrol agents in timber preservation (Schoeman et al., 1996; Wheatley et al., 1997). Some sesquiterpenes may be involved in defense responses against fungivores (Wiens et al., 1991; Stadler and Sterner, 1998).

Most studies have investigated the identities of VOCs produced as a snapshot at a single time point; however, the VOC profile can change over time (Sunesson et al., 1995; Nilsson et al., 1996; Korpi et al., 1999). For example, volatile production by *Penicillium* growing in pure culture reached a peak after 4–5 d (after normalization for colony size; (Nilsson et al., 1996). When mycelia are interacting, production of VOCs is likely to change considerably during different stages of the interaction. The current study follows VOC production over the time course of mycelial interaction between two wood decay basidiomycetes, *Hypholoma fasciculare* and *Resinicium bicolor*. Although the latter is most common in coniferous woodlands, they are both found in angiosperm woodland, where their mycelia could potentially interact while growing across soil or within wood (Kirby et al., 1990; Boddy, 2001).

Sampling was timed to coincide with different morphological manifestations of interaction: on agar, growth usually slows following hyphal contact, red/brown pigment is produced by *R. bicolor* in the interaction zone, while *H. fasciculare* forms cords following mycelial contact that produce a yellow pigmentation approximately 10 d later, ultimately with slow mutual replacement (Fig. 1). The following hypotheses were tested: (1) VOCs are produced prior to mycelial contact, initiating an early defense response; (2) VOC production is linked to the production of pigments during interspecific interaction; (3) VOC production increases over the course of the interaction independently of colony size; and (4) interspecific interactions produce additional VOCs, which are otherwise not produced during self interactions.

One means of sampling headspace VOCs is to use solid phase microextraction (SPME) fibers. The fiber coatings have different properties for adsorption of different polarity compounds (Nilsson et al., 1996; Jeleń, 2003), and both polydimethylsiloxane (PDMS) and polyacrylate fibers have been used successfully to sample fungal VOCs (Nilsson et al., 1996; Fäldt et al., 1999; Jeleń, 2003; Demyttenaere et al., 2004; Ewen et al., 2004). SPME PDMS sampling, combined with separation and identification by GC/MS, was used to monitor the production of VOCs emitted from interactions of *H. fasciculare* and *R. bicolor* grown over 0.5% malt extract broth.

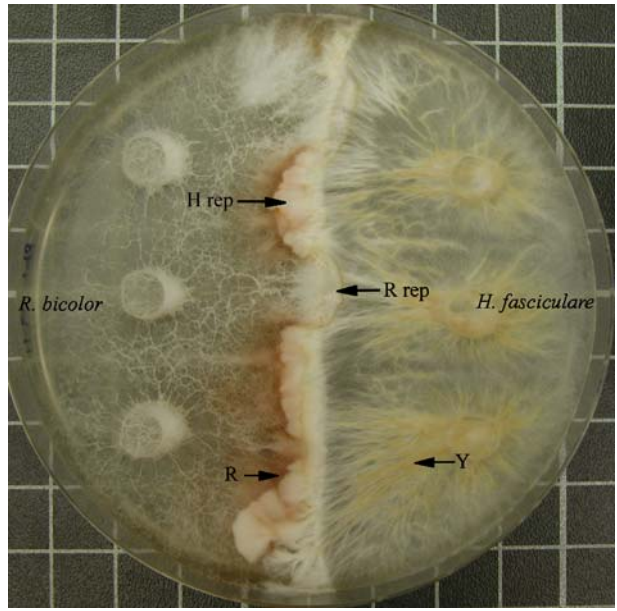


Fig. 1 *Resinicium bicolor* interacting with *Hypholoma fasciculare* on 2% malt agar in a 9-cm Petri dish, 18 d after mycelial contact at 20°C. *Resinicium bicolor* is partially replacing *H. fasciculare* (*R rep*) and *vice versa* (*H rep*). Note that there is dark red/brown pigment production by *R. bicolor* (*R*), and yellow pigment production around cords of *H. fasciculare* (*Y*). Photograph courtesy of Tim Rotheray

Methods and Materials

Fungi

Cultures of *H. fasciculare* (HF-DD2) and *R. bicolor* (RB1), from the Cardiff University Fungal Ecology Group culture collection, were initially grown separately on 2% malt extract agar (Lab M MC23 malt extract, Lab M agar no. 2) in 9-cm nonvented Petri dishes at 20°C to provide fungal inoculum. Inocula comprised plugs of mycelium plus agar (10 mm diameter) cut from the margin of growing cultures with a no. 5 cork borer. Growth rates of the two species were similar; thus, in interaction systems (see below), both plugs were added at the same time.

Interaction Systems

Interactions took place in Reacsyn™ fermentation vessels (BioDiversity, Enfield, UK)—polypropylene bottles containing a porous polyester/polypropylene support at the base of an inner chamber (Fig. 2). The support sat in contact with the surface of the broth medium [30 ml of sterile malt extract broth (0.5%, Lab M MC23 malt extract)] contained in the outer chamber. A Fluropore™ membrane (1 μm pore size; Millipore, Watford, UK) was placed onto the vessel support, and fungal inocula were placed on or near the center (see below). The membrane, although not completely preventing growth into the culture medium, encouraged horizontal growth of the mycelium ensuring a physical interaction (similar to that observed on solid agar plates) and provided a stark white background against which any hyphal pigmentation could clearly be seen. The neck of the inner chamber was stoppered with a gas permeable polyurethane foam bung. The inner/outer chamber design allowed simple sampling of both the headspace (40 ml) and broth (30 ml) within an aseptic system.

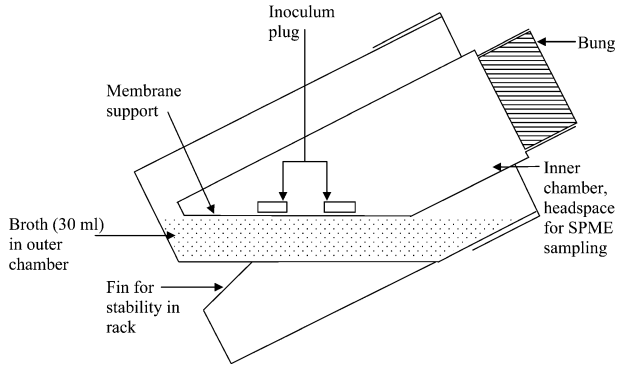


Fig. 2 Diagram of the Reacsyn™ fermentation vessels used to assess production of volatiles and diffusibles when mycelia were interacting and when growing alone

Treatments

Five treatments were prepared in triplicate, two single plugs of each isolate, two self pairings, and one interspecific interaction: (1) a single *H. fasciculare* plug inoculated centrally (HF), (2) two *H. fasciculare* plugs placed 10 mm apart (HFHF), (3) a single *R. bicolor* plug inoculated centrally (RB), (4) two *R. bicolor* plugs placed 10 mm apart (RBRB), and (5) one *H. fasciculare* plug placed 10 mm away from a single *R. bicolor* plug (HFRB). The vessels were placed in their commercial racking system and incubated in the dark at 20±0.5°C.

Sampling of Volatiles

Volatiles were collected by SPME (100 μm PDMS fiber, Supelco, Poole, Dorset, UK). Guided by a small preliminary experiment, sampling took place 1, 3, 9, 17, and 25 d after inoculation. The first sample of the volatiles, the baseline, was taken the day after inoculation, allowing the dispersal of any compounds produced or released as a result of mycelial severance. The 3-d sample was prior to mycelial contact with about 1.5–2-mm distance between the colonies. *Resinicium bicolor* started to produce pigment at 7–8 d; thus, pigment production was initiated 1–2 d prior to sampling at 9 d. The last two time samplings represented later stages of the interactions. The SPME sampling process was as follows: the SPME fiber (preconditioned according to the manufacturers' instructions) was briefly conditioned at the start of sampling each day (250°C, 15 min) and a fiber blank was then run. The inoculated Reacsyn vessels were flushed to clear any build up of fungal volatiles and plasticizer by placing in a sterile laminar flow system with the foam bung/cap system removed for 10 min (deemed suitable following preliminary trials). The cap was then replaced and the vessel was removed from the flow. The SPME fiber was pushed through the bung and exposed to the headspace of the inner chamber for 1 h. The SPME fiber was withdrawn and immediately injected into the GC/MS system.

GC/MS Analysis

Volatiles were analyzed by GC (GC8000, Thermofinnigan, Hemel Hempstead, UK) and MS (MD 800, Thermofinnigan). Samples were desorbed at 220°C in a split/splitless injection port in splitless mode for 2 min and resolved over 30 m VF23ms (Varian, Palo Alto, CA, USA) polar column (0.25 mm ID, 0.25 μm film thickness), with helium carrier

gas (55 KPa). The temperature program was as follows: start 45°C, increasing at 3°C/min to 200°C, then held at 200°C for 5 min. Mass spectra were recorded in electron ionization mode from m/z=35 to 400, with a source temperature of 200°C and interface at 280°C.

Fiber blanks were used to check for any signs of contamination. Control Reacsyn vessels containing malt broth but no fungus were tested by SPME for any exchange of volatiles between the vessels being incubated together. Putative identifications were made by comparing mass spectra with library entries (National Institute of Standards and Technology v2.1 mass spectral database). An absolute identification would have required preparation and/or provision of individual reference compounds and was beyond the scope of this project. Peaks of interest were integrated by using Masslab v1.4 (Thermofinnigan). Prior to statistical analysis, peak areas for each time point were normalized against dibutylbenzene (plasticizer signal, RT 11.9), which remained constant across all samples throughout the time course of the experiment. Peaks found in only one replicate of a treatment were reported but considered artifacts and were not included in further analysis. The evolution of each volatile (as peak area, in standard equivalent units, over time) was compared by using one-way analysis of variance or two-sample *t*-test (Minitab v13) by comparison of area under the curve determined by KaleidaGraph software (Synergy Software, Reading, PA, USA).

Results

Visible Changes

Mycelial morphology changed after mycelial contact. Red/brown pigment was produced beneath mycelium of *R. bicolor* between 3 and 9 d, and yellow pigment developed in *H. fasciculare* cords between 9 and 17 d.

Between Treatment Differences in Volatiles

Replicates within each treatment were generally consistent (and consistent with the preliminary experiment), although one replicate from the interspecific interaction produced the same volatile peaks as the other two replicates but at lower concentrations. In total, 25 fungal metabolites were detectable in the five treatments (Fig. 3). The interspecific interaction produced 12 volatiles that were not found in the single species samples (peaks 1–4, 6, 7, 9, 14, and 21–23), and additionally three (peaks 8, 12, and 17) were considered to be produced only during interspecific interaction because they were present only in a trace amount in a single replicate of the single-species samples. Four peaks were common to the interspecific interaction and *R. bicolor* samples (peaks 5, 11, 16, and 19), three to the interspecific interaction and *H. fasciculare* (peaks 10, 24, and 25), one peak was unique for *R. bicolor* (peak 13), and three peaks (peaks 15, 18, and 20) were identified in all treatments. Peak 20 was produced in significantly higher concentration by *R. bicolor* than *H. fasciculare* ($P<0.05$). An additional peak (peak 23), although only present in a single replicate of the interspecific interaction (suggesting it was not a consistently produced volatile), was included in the total peak count, as it was putatively identified as a sesquiterpene. The majority of the metabolites produced were determined to be sesquiterpenes eluting between 12.5 and 21 min (peaks 1–17). Two further sesquiterpenes eluted at 29.1 and 33.9 min (peaks 19 and 23). Two sets of diastereoisomers were putatively

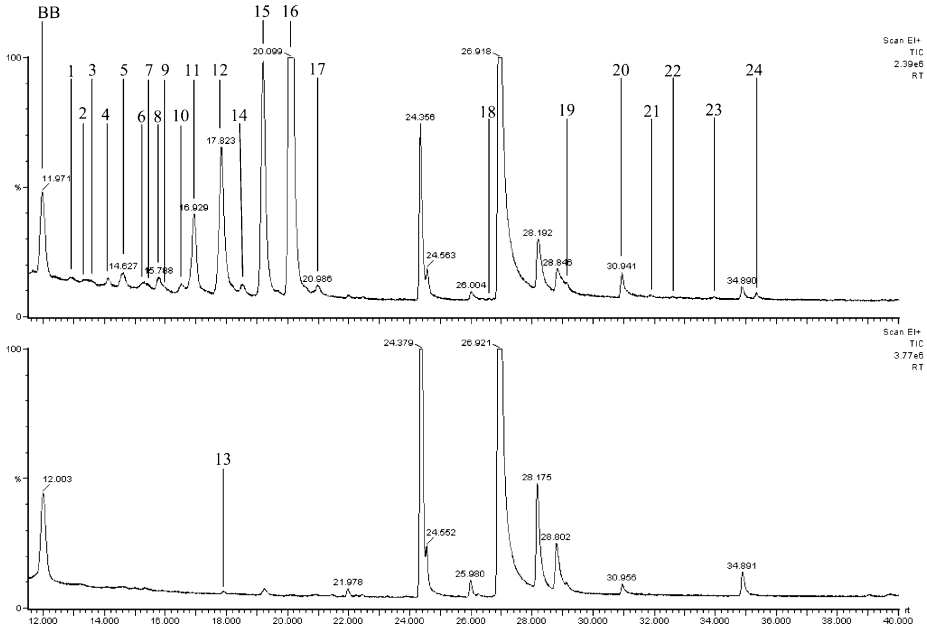


Fig. 3 Representative GC/MS traces indicating fungal metabolites: **(a)** *Hypholoma fasciculare* v *Resinicium bicolor* (HFRB) interaction at 17 d; **(b)** *R. bicolor* (RBRB) alone at 17 d indicating position of the peak found in *R. bicolor* only. Peaks are numbered individually. An additional small peak (25) was found in both treatments at 49.1 min (not shown), *BB* is dibutylbenzene

identified from mass spectral data, peaks 3, 5, 14, and 16 being γ-muurolene, γ-cadinene, γ-amorphene, and γ-bulgarene, and peaks 4, 11, 15, and 17 being α-muurolene, α-cadinene, α-amorphene, and α-bulgarene, although the order of elution of the isomers could not definitely be assigned due to the similarity of the mass spectra within each set. Peak 12, although a major peak, did not produce any matches in the database, so it was not identified. Of the remaining peaks, peak 18 appeared to be a benzoic acid methyl ester, peak 20 a benzyl alcohol, and peak 24 a quinolinium-type compound with a distinctive fragmentation pattern at *m/z* 203, 204, 206, and 207. The remaining peaks, peaks 21, 22, and 25, had *m/z* maxima of 163, 159, and 206–208, respectively, but could not be identified.

In most cases, the production of specific volatiles was not affected by colony size (no significant differences found between single- and double-plug single-species treatments, $P > 0.05$). Peaks 11 and 16, however, were detected in the double- but not in the single-plug *R. bicolor* treatment, and peak 19 also indicates some effect of colony size—HFRB being significantly different ($P \leq 0.05$) from RB but not RBRB (Fig. 4). Where volatiles were

Fig. 4 Evidence of up-regulation of VOCs during interspecific interaction in those volatiles that were also present in single species inoculations: *HF*, *Hypholoma fasciculare* inoculated singly; *HFHF*, *H. fasciculare* inoculated as two plugs; *HFRB*, *H. fasciculare* v *Resinicium bicolor*; *RB*, *R. bicolor* inoculated singly; *RBRB*, *R. bicolor* inoculated as two plugs. Values are means (with standard errors) of three replicates of total area under the curve produced over 25 d. For each peak, the same letter indicates no significant difference ($P \leq 0.05$). Log_{10} transformation of data were required for peaks 10, 11, 15, 16, and 20. One-way ANOVA with Tukey–Kramer *a posteriori* test performed on 5, 10, 15, 18–20, and 24. Two-sample *t*-test was carried out on peaks 11 and 16

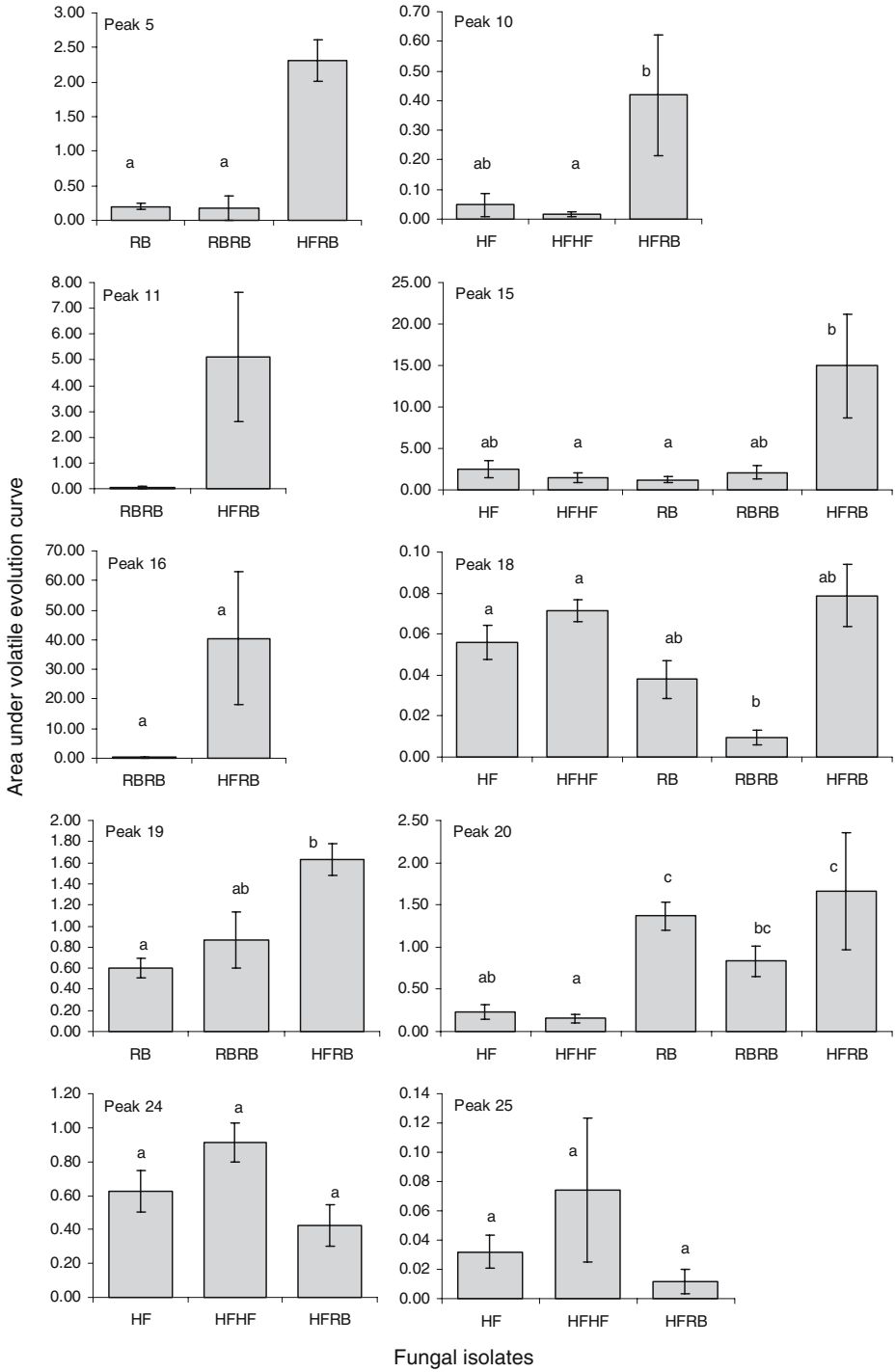

Peak 5
Peak 10
Peak 11
Peak 15
Peak 16
Peak 18
Peak 19
Peak 20
Peak 24
Peak 25
Area under volatile evolution curve
Fungal isolates
RB
RBRB
HFRB
HF
HFHF

Table 1 Comparison of the evolution of fungal volatiles graded on a log scale for peak area (standard equivalent units) allowing comparison of peak areas over time and between peaks

Peak Number	Ret. Time	Days						
		HF					HFvHF	
		1	3	9	17	25	1	3
1	12.9	*	*	*	*	*	*	*
2	13.29	*	*	*	*	*	*	*
3	13.50	*	*	*	*	*	*	*
4	14.1	*	*	*	*	*	*	*
5	14.6	*	*	*	*	*	*	*
6	15.3	*	*	*	*	*	*	*
7	15.40	*	*	*	*	*	*	*
8	15.8	*	*	*	*	*	*	*
9	16	*	*	*	*	*	*	*
10	16.5	*	*	*	0.01**	*	*	*
11	16.9	*	*	*	*	*	*	*
12	17.8	*	*	*	*	*	*	*
13	17.9	*	*	*	*	*	*	*
14	18.5	*	*	*	*	*	*	*
15	19.2	*	*	0.03***	0.22****	0.13***	*	0.00*
16	20.09	*	*	*	*	*	*	*
17	20.9	*	*	*	*	*	*	*
18	26.5	0.00*	0.00**	0.00**	0.00**	0.00**	0.00*	0.00**
19	29.15	*	*	*	*	*	*	*
20	30.9	*	0.00*	0.02***	0.01**	*	0.00*	0.00**
21	31.89	*	*	*	*	*	*	*
22	32.65	*	*	*	*	*	*	*
23	33.97	*	*	*	*	*	*	*
24	35.35	0.02***	0.02***	0.03***	0.04***	0.01***	0.04***	0.08***
25	49.18	*	*	0.00*	0.00**	*	*	*

Log scale for peak area (standard equivalent units) *<0.001; **=0.001–0.01; ***=0.01–0.1; ****0.1–1; *****>1

present in both the interspecific interaction and other treatments, up-regulation tended to occur, the area under the volatile evolution curve from the interspecific interaction being greater ($P \leq 0.05$) than both single- and double-plug isolate samples for peaks 5 and 11 RBRB (Fig. 4). In the case of peaks 10, 15, and 16, the large amount of variability within the interspecific interaction, which was caused by the outlying response of replicate 1, resulted in a lack of significant difference between some samples (Fig. 4). No up-regulation occurred in the production of the metabolites corresponding to peaks 18, 20, 24, and 25.

Production of Volatiles Over Time

All VOCs detected increased over the time course of the experiment and then declined. Of the 25 metabolites, only four were detected at 1 d (Fig. 4, Table 1): peaks 18, 19, 20, and 24 from 4, 1, 4, and 9 samples, respectively; peak 24 being the only metabolite consistently present at 1 d. Production of other metabolites began between 3 and 17 d, but most commonly from 9 d following mycelial contact (Table 1), and increased over time, most reaching a maximum at 17 d and declining by 25 d (Table 1, Fig. 5).

Table 1 (Continued)

Days								
HFvHF			RB					RBvRB
9	17	25	1	3	9	17	25	1
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	0.03***	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	0.0**	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	0.00**	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
0.04***	0.13****	0.04***	*	*	0.00**	0.14***	0.02***	*
*	*	*	*	*	*	0.00**	*	*
*	0.01**	*	*	*	*	*	*	*
0.00**	0.00**	0.00**	0.00**	0.00*	0.00**	0.00*	0.00**	*
*	*	*	*	*	0.04***	0.04***	0.01**	0.00**
0.02***	0.00*	*	*	0.00**	0.08***	0.09***	0.02***	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
0.04***	0.02***	0.02***	*	*	*	*	*	*
0.00**	0.01**	*	*	*	*	*	*	*

Discussion

There was no evidence of long-distance antagonism (Boddy 2000) in the interaction between *H. fasciculare* and *R. bicolor*; morphological responses were not manifest until after mycelial contact, and there was no physiological response (as evidenced by lack of change in VOC profiles) until 8 d after inoculation. By this time, mycelial contact had been made, and *R. bicolor* had begun to produce red/brown pigments (3–9 d). Those \VOCs emitted before 9 d (peaks 4–6, 8–10, 13–15, 17–20, 22–24, and 25) may relate to *R. bicolor* pigment production, while those which did not occur until after 9 d (peaks 1–3, 7, 11, 12, 16, and 21) may be associated with the yellow pigment production seen in the mycelium of *H. fasciculare* at this time. Shifts in metabolism corresponding with pigment production have also been detected in *Phlebia radiata* (Griffith et al., 1994a; Rayner et al., 1994).

The decline in VOCs concentration towards the end of the experiment may have several causes. First, because there was no overall replacement of one fungus by the other, there was no new mycelial contact to induce fresh VOC production. Continued production of VOCs might be envisaged where one fungus replaces another with the production of

Table 1 (Continued)

Days								
RBvRB				HFvRB				
3	9	17	25	1	3	9	17	25
*	*	*	*	*	*	*	0.01***	0.00**
*	*	*	*	*	*	*	0.02***	0.00**
*	*	*	*	*	*	*	0.00**	*
*	*	*	*	*	0.00**	0.03***	0.05***	0.00**
*	0.02***	*	*	*	*	0.12****	0.19****	0.00*
*	*	*	*	*	*	0.02****	0.33****	0.00**
*	*	*	*	*	*	*	0.03***	*
*	0.04***	*	*	*	*	0.01**	0.09***	0.03***
*	*	*	*	*	*	0.00**	0.03***	0.00**
*	*	*	*	*	*	0.00**	0.05***	0.01**
*	0.00**	0.00**	*	*	*	0.03***	0.56****	0.10****
*	*	*	*	*	*	0.07***	1.02*****	0.18****
*	0.02***	0.01***	*	*	*	*	*	*
*	*	*	*	*	*	0.09***	0.04***	0.00*
*	0.13***	0.13***	0.03***	*	0.00**	0.18****	1.56*****	0.32****
*	0.02***	0.01***	*	*	*	0.02***	4.62*****	0.84****
*	*	*	*	*	*	0.00**	0.03***	0.01**
0.00*	0.00*	0.00*	0.00*	*	0.00*	0.00**	0.01**	0.00**
*	0.07***	0.04***	*	*	*	0.12****	0.09***	0.03***
0.01***	0.06***	0.04***	0.01**	0.00*	0.01***	0.06***	0.13****	0.03***
*	*	*	*	*	*	0.00*	0.01**	*
*	*	*	*	*	*	0.01**	0.01**	*
*	*	*	*	*	*	0.00**	0.00**	*
*	*	*	*	0.02***	0.02***	0.02***	0.02***	0.01**
*	*	*	*	*	*	0.00*	0.00**	*

successive bands of pigment by either or both the invading and invaded fungus. In other fungal combinations, where pigment production is less, there may be less VOCs produced even during the height of the interaction. Second, the nutrient limitations of the media may have prevented further metabolite production as the number (Sunesson et al., 1995; Korpi et al., 1999) and concentration (Demyttenaere et al., 2004) of VOCs produced can vary depending upon the growth medium.

The outcome of an interaction may depend to some extent on the ability of a fungus to detoxify its opponent's metabolites. Catabolism or detoxification of VOCs may have occurred during the current study; at least one compound produced by *R. bicolor* grown on its own was not detected during the interspecific interaction. This may indicate the inhibition or switching off of some metabolites during interaction. Also, there was some evidence that plasticizer (at midretention times) from the Reacsyn vessels decreased with time, perhaps due to fungal metabolism. Evidence that *H. fasciculare* does breakdown diffusible compounds produced by other fungi was provided by Griffith et al. (1994b): *H. fasciculare* had a reduced radial extent when inoculated onto a cellulose film overlaid onto agar on which an opposing species had initially been grown; when this cellulose film plus fungus was removed and replaced by a fresh inoculum of *H. fasciculare* on a fresh cellulose film, the latter grew much more extensively, indicating that some degradation of

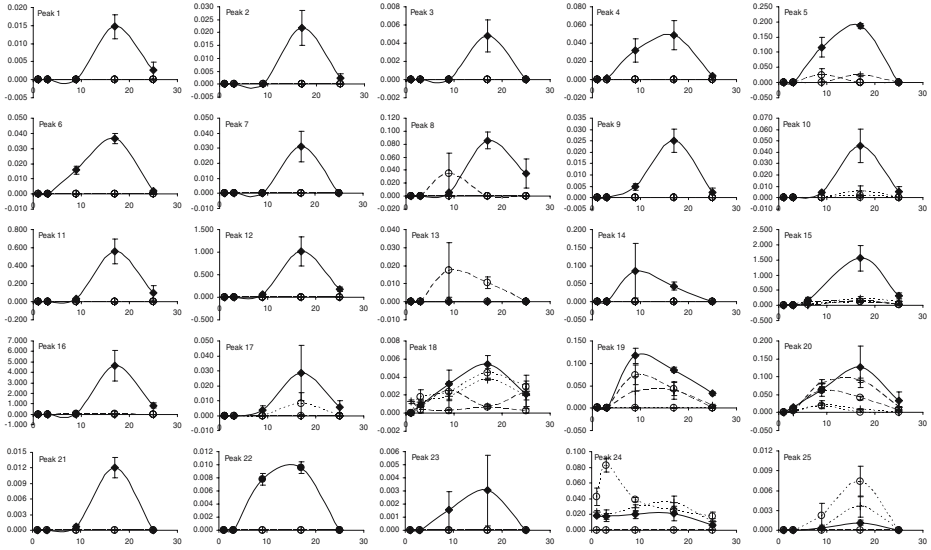


Fig. 5 Production of each volatile (Peaks 1 to 25) in all five treatments over time, values are means (with standard errors) of three replicates. *Dotted lines* HF, *open circles on dotted lines* HFHF, *dashed lines* RB, *circles on dashed lines* RBRB, *solid diamonds* HFRB

the inhibitory compounds had occurred during the growth of the initial *H. fasciculare* inoculum.

Some VOCs were produced as part of the normal metabolism of each fungal isolate, but may still have a role in the defense of territory against invasion by other fungi. Wood previously colonized by decay fungi is inhibitory (and occasionally stimulatory) to other fungi even when the original occupants are dead (Heilmann-Clausen and Boddy, 2005). VOCs may be up-regulated during interaction, and in the current study, two VOCs (peaks 5 and 11) were up-regulated to a statistically significant extent ($P \leq 0.05$) during the interaction compared with the fungi growing alone. There was also some indication that peaks 10, 15, 16, and 19, may be up-regulated during interaction. These four peaks, which were not specific to the mixed species interactions, were some of the nonsesquiterpene compounds detected.

Of the 25 VOCs detected when the fungi were growing alone and during interaction, 19 were sesquiterpenes. Sesquiterpenes are widely used for communication among invertebrates, used by invertebrates to locate plant or animal hosts and by plants and fungi to attract insects for propagule dispersal. Many plants (Cakir et al., 2005; Cheng et al., 2005; Wang et al., 2005) and fungi (Florianowicz, 2000; Roy et al., 2003) produce sesquiterpenes with antifungal activity. An increase in sesquiterpene concentration including that of γ-muurolene has been found in response to wounding and fungal inoculation of spruce, suggesting that sesquiterpenes are part of an antifungal response (Viiri et al., 2001). Other sesquiterpene extracts show antifeedant activity or are ultimately toxic to invertebrates (Wiens et al., 1991; Kahlos et al., 1994; Stadler and Sterner, 1998). α- and γ-muurolene and γ-cadinene have been detected in red and white cabbage and nasturtium infested with *Pieris brassicae* or *Pieris rapae* larvae, but not in uninfested plants, potentially forming part of an antifeedant plant defense response (Geervliet et al., 1997).

On the other hand, cadinenes, muurolenes, and amorphenes, putatively identified in our samples, are also used in insect communication systems (El-Sayed, 2005); for example, α-

and γ-muurolene contribute to the attraction of females of the moth, *Helicoverpa armigera*, to pigeon-pea plants (Hartlieb and Rembold, 1996). Germacrene D, an important precursor to the cadinenes, muurolenes, and amorphenes (Bulow and König, 2000), also strongly affects insect behavior (Prosser et al., 2004), activating an antennal response (Røstelien et al., 2000; Mozuraitis et al., 2002), and increases attraction to the substrate and oviposition by the tobacco budworm moth *Heliothis virescens* (Mozuraitis et al., 2002). The precise effect of the volatiles, produced during interspecific fungal interactions, on invertebrate behavior is, however, not yet known.

The two sets of putatively identified diastereoisomers (α- and γ-muurolene, cadinene, amorphene, and bulgarene, peaks 3, 4, 5, 11, 14, 15, 16, and 17) were generally associated with the interspecific interaction, although peaks 5, 11, and 16 were also found in RB treatments. The fact that peaks 11 and 16 occurred in the RBRB interaction but not RB only suggests some interaction response even with self isolates. General inducement or upregulation of compounds during the HFRB interaction suggests that they are part of non-self recognition and/or defense process, which is also visually manifest in the development of pigments from both species and the reduction of growth towards each other. Initial experiments with collembola also indicate attraction to certain regions of fungal growth, and toxicity of the pigmented cords of *H. fasciculare* (T.D. Rotheray, personal communication).

The preponderance of sesquiterpenes in the VOC profile should, however, be viewed with some caution as SPME fibers are particularly good at absorbing sesquiterpene hydrocarbons (often reported as fungal VOCs), and the PDMS-coated fiber absorbed the highest proportion of sesquiterpenes when compared against three other fiber coatings (Jeleń, 2003). Not all compounds can be picked up by SPME sampling. Sesquiterpene oxygenated compounds were completely missed by SPME sampling, while they were detected by simultaneous distillation extraction. However, other compounds that were detected by SPME were missing from the simultaneous distillation extraction (Jeleń, 2003). Other extraction techniques such as absorption on tenax followed by thermal desorption and solid sample injection (SSI) may offer a more comprehensive extraction. Other extraction techniques also have their disadvantages with respect to, for example, time for preparation and losses by degradation during the introduction of the analyte to the GC column in the case of SSI, and the decomposition of certain compounds on tenax (Ewen et al., 2004). In summary, although SPME sampling by PDMS fiber may provide an incomplete selection of the VOCs released by the fungi, it allows for rapid simple nondestructive sampling, and has provided a sufficient selection of compounds to elucidate the changes in metabolism associated with fungal interactions.

Of the four hypotheses tested, hypothesis 1, that VOCs are produced prior to mycelial contact initiating an early defense response, was not supported. Findings, however, supported the other three hypotheses: VOC production was linked to the timing of pigment production and may even provide a way of separating the metabolites produced by each species where the pigments are produced at distinctly different times (hypothesis 2). Some VOCs produced by the fungi when growing alone and during the interspecific interaction were significantly up-regulated during the interaction (hypothesis 3), while, compared with the individual fungi, a new set of VOCs was produced during the interspecific interaction (hypothesis 4).

Future priorities are further identification of volatiles and diffusibles exuded during fungal interactions. This will also involve determining whether or not the VOCs are attractive and/or toxic to insects and inhibitory to other fungi. We need to link volatiles/

diffusibles back to genes and to determine if genes are being up-regulated. The function of the pigmentation and the relationship between pigmentation and VOC production must be determined. The work also needs to be extended to combinations of fungal species beyond those used here. Different species of fungi are likely to produce different sets of metabolites when growing alone and during interaction, although there is some crossover in the metabolites produced.

Acknowledgements We thank the Natural Environment Research Council for funding (003/00731), John Hedger, the Cardiff University Fungal Ecology Group, and Tim Rotheray for discussion and for Fig. 1.

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J Chem Ecol (2007) 33:59–73
DOI 10.1007/s10886-006-9203-z

Kairomones Extracted from Rice Yellow Stem Borer and their Influence on Egg Parasitization by *Trichogramma japonicum* Ashmead

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Received: 27 January 2006 / Revised: 15 August 2006 / Accepted: 5 September 2006 /
Published online: 5 December 2006
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Abstract Host location and acceptance by egg parasitoids can be mediated by close-range host stimuli. In this study, we tested the response of *Trichogramma japonicum* Ashmead to cuticular extracts of adult and larval rice yellow stem borer (YSB), *Scripophaga incertulas* Walker. We also studied the wasps' response to extracts from YSB larval frass. Laboratory bioassays revealed that hexane extracts of the adult host body stimulate ovipositor probing of *T. japonicum*. Extracts of larval frass also stimulated parasitization. In contrast, host larval cuticular extracts had no effect on parasitization rates. Fractionation of the crude extracts of adult YSB cuticular extracts was performed using silica gel chromatography, followed by bioassays of the individual fractions to test their effects on wasp behavior. Analyses of the most active fractions by gas chromatography–mass spectrometry revealed that the extract contained saturated long chain alkanes and alkenes, with carbon numbers ranging from C_{20} to C_{32}. Hydrocarbons were applied onto host eggs to test their effects on parasitization rates. Treatments of eggs with docosane, tetracosane, pentacosane, and eicosane enhanced host egg parasitization, while pentadecane, hexadecane, and nonadecane deterred oviposition.

Keywords Rice yellow stem borer · *Scripophaga incertulas* Walker · Oviposition · Parasitoid · Cuticular extracts

Introduction

Parasitoids of phytophagous insects are known to orient to plants on which their hosts are found. They often use plant- and host-derived chemicals that indicate or confirm the

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presence of the host (Vinson, 1976). Chemicals emanating from the host insect or the plant itself may arrest parasitoids in the vicinity of their hosts, thus mediating successful host location and parasitization (Gross et al., 1984). During host finding, two types of stimuli may operate: "attractant" stimuli that elicit orientation to areas with hosts and "arrestant" stimuli that elicit a reduction in the distance or area covered per unit time by parasitoids moving within this area (sensu Dethier et al., 1960). The stimuli influencing searching, parasitization, or retention of the parasitoids originate from various sources, such as host food source, the host itself, or host body emissions (Turlings et al., 1995; Rutledge, 1996; Dorn et al., 1999). In parasitoid–host relationships where kairomones are involved in host location, the duration of parasitoid patch visits is often mediated by, e.g., a host kairomone, host contact cues that may affect the handling time, host density, and rate of oviposition (Strand and Vinson, 1982). Waage (1978) and Weseloh (1980) found that an increasing concentration of kairomone is an indication of increased host density that prolongs the parasitoid's patch arrestment.

Among host-related materials, scales from adult hosts display kairomonal activity towards several parasitoid species. For example, Lewis and Jones (1971) demonstrated that parasitization by *Trichogramma evanescens* was higher for eggs placed in areas contaminated with moth odor. Scales left by the moths were suspected as the source eliciting parasitization (Lewis et al., 1972). In addition to volatile semiochemicals, nonvolatile kairomones also mediate searching behavior of *Trichogramma* spp. Plants on which adult moths have been present may provide cues for host location. Furthermore, egg deposition by herbivorous insects can induce changes of the plant that attract or arrest parasitoids (Hilker et al., 2000; Hilker and Meiners, 2006).

Larvae of the yellow stem borer (YSB) *Scripophaga incertulas* are a major agricultural pest on rice in India and several other countries in Asia. A single female lays about 2–3 egg masses on the tip of the upper surface of tender leaf blades and covers them with hairs and scales from its anal tuft. The larvae enter the leaf sheaths, feed for 2–3 d, and bore into the stem near the nodal region (Pathak and Khan, 1994). They infest plants during their entire larval development. Rice plants, from seedlings to maximum tillering stages that have been attacked at the base of the stem, have dead hearts on the dried up central shoots of tillers. The rice YSB is monophagous on rice, and larvae may feed within the stem without causing severe damage to growing plant parts at the base. However, larval feeding can result in reduction of plant vigor (van Vreden and Ahmadzabidi, 1986). Before pupation, a larva covers the exit hole with thin webbing. Often, larval frass is visible at this exit hole. The larvae and pupae that cause the most damage are concealed inside the stem. Hence, these pests are difficult to control. Traditionally, YSB has been controlled primarily by repeated application of pesticides. In recent years, as the awareness of the economic costs of pesticides and their ecological impact on the environment has increased, biological control by means of parasitoids and predators has been used with varying degrees of success in many different areas (Perez and Cadapan, 1986).

Trichogramma japonicum is an important egg parasitoid of YSB in southern India. It parasitizes the egg masses of YSB that are laid on rice leaves. The mechanisms by which *T. japonicum* locates YSB on rice have not been investigated. In this paper, we demonstrate that chemicals originating from adult hosts mediate close range orientation and oviposition by *T. japonicum* females in host eggs, thereby enhancing the level of parasitization. An attempt was also made to isolate the active chemicals by fractionation and to identify them. Comparisons of the oviposition stimulatory activity, as well as the nature of the chemicals present in the host adult cuticular extracts and frass, were made with that of the larval body

extracts. Extracts of the YSB larval body material were used only for comparison. As internal feeders, larvae might not emit any attractive volatiles.

Methods and Materials

Insects

Plants infested with the YSB *S. incertulas* were collected from Rice Research Station, ANGR Agricultural University, Rajendranagar area of Hyderabad, India, and maintained in the laboratory glass house. Rice YSBs were reared on potted rice plants, Samba masoori variety. Rearing conditions were 30±2°C under natural day light conditions. Pure cultures of *T. japonicum* were obtained from the Project Directorate of Biological Control, Bangalore, India, where they were reared on eggs of a factitious host, *Corcyra cephalonica* Stainton (rice moth) (Lepidoptera: Pyralidae). *Corcyra cephalonica* was reared on sorghum grain. Female parasitoids used for the experiments were 1–2 d old, mated, naïve with respect to the host-related volatiles, and fed with 1:1 water-diluted honey.

Adult YSB were collected from the rearing cages before each experiment and were killed or immobilized by placing them for a few minutes into the refrigerator. Stems of rice plants were cut open to collect frass and 5th instar larvae separately, followed by immobilization of the larvae in the refrigerator.

Extraction

YSB adults and larval body washings were obtained by placing immobilized moths and larvae of known weight in small conical flasks (50 ml) containing analytical grade hexane (2 g per 10 ml that is 18 AE/ml) and shaking them vigorously at a room temperature of 24± 12°C for 2 min. Adults were 2–4 d old. Larval extracts were taken from 5th instars. The washings were filtered through a Whatman no. 1 filter paper. The extract was subsequently concentrated by vacuum evaporation and stored at −20°C. Extracts of frass of 5th instars were obtained in a similar manner.

Isolation and Identification

Concentrated crude extracts were separated using SiO_2 as adsorbent in a 25-mm-diameter column. The extract (0.5 ml) was loaded onto a column (1.5×50 cm) containing 20.0 g silica gel (200 mesh) and was eluted consecutively with 300 to 500 ml of solvent of increasing polarity. Elution was carried out gradually with hexane–ethyl acetate in the following combinations: 100, 98:2, 96:4, 94:6, 92:8, 90:10, 88:12, and 80:20. Analytical-grade solvents were used. Several fractions were collected, and their homogeneity was monitored by using thin layer chromatography. Resulting fractions were reduced to 1.0 ml by passing N_2 gas to avoid loss of volatile monoterpenes, which were significant components of these fractions as indicated by gas chromatography–mass spectrometry (GC–MS) analyses.

All fractions were subjected to bioassays with *T. japonicum*. Bioactive compounds were selected for further chemical studies. Semipurified fractions of each extract were separately bioassayed, and the active fraction was subjected to GC and GC–MS identification.

Certain hydrocarbon compounds were found in the extracts of YSB-related materials by GC and GC–MS (see below). To study their kairomonal activity to the parasitoids, hexadecane, eicosane, docosane, pentacosane, tetracosane, tricosane, pentadecane, nonadecane, heneicosane, and hexacosane (99% pure) were purchased from Sigma Chemicals, St. Louis, MO, USA, and tested.

Gas Chromatography

Each fraction (1 μl) was injected into a GC (Agilent 6890 series) with a HP-1 capillary column (30.0 m long×320 μm i.d.×0.25 μm film thick). The GC was temperature programmed from 100°C to 300°C at 10°C/min. Nitrogen was the carrier gas, and the flow was fixed at 2 ml/min. Injection temperature was 150°C.

Gas Chromatography–Mass Spectrometry

The concentrates of the hexane–ethyl acetate eluate of the extracts were injected into an Agilent 6890 GC equipped with a 5973N model mass selective detector (MS). A HP 5 MS (Hewlett Packard, Palo Alto, CA, USA) capillary column (30 m long×250 μm i.d.× 0.25 μm film thick) was used. The column oven was programmed after an initial delay of 2 min from 50°C to 300°C at 10°C/min. Helium was used as carrier gas with a flow rate of 1.2 ml/min. The sample (1 μl) was introduced in split ratio of 10:1 at an injection temperature of 150°C.

The chemical structure of each compound was elucidated by comparison of the mass spectra with those of authentic chemical samples or by comparison with data in the mass spectral library of the Analytical Division, Indian Institute of Chemical Technology. Relative amounts were calculated from the chromatogram peak area.

Testing Effects on Parasitization Rates

To study the effect of different samples on parasitization rates, the surfaces of host eggs were treated with either a test solution or solvent. Laboratory assays were conducted as tests following the methods used by Jones et al. (1973) and Ananthakrishnan et al. (1991). Responses of *T. japonicum* to the stimuli offered were measured in terms of the number of eggs parasitized. A circle of Whatman no. 1 filter paper (150 mm diameter) was divided into eight sections by lining with a pencil and then fitted to the bottom of a plastic petri dish (150 mm diameter). About 50 eggs of *C. cephalonica* were glued to white card squares (1× 1 cm). Such a card was placed in the center of each section and stuck to the filter paper lining with a tiny drop of light, odorless adhesive (Camlin India, Mumbai, India). Eight groups (cards) of eggs were placed in a single dish.

Dual choice tests were conducted. Parasitoids were exposed to host eggs treated with chemical and solvent-treated control simultaneously in a single petri dish. The test compound (i.e., 20 μl of the extract, chromatographic fraction, or synthetic hydrocarbon) was applied to each of the four pieces of egg cards and placed in four opposite sections. Solvent-treated controls were placed in the remaining four sections, so that each treated egg card was placed between two control cards.

In a second series of multiple choice tests, eight egg cards, each containing 50 eggs, were placed diagonally opposite to one another, and the surfaces of two opposite egg cards

were treated with a fraction. The preference of parasitoids was measured by giving them a choice of four test samples.

For both dual and multiple choice tests, glass tubes (3.7 cm diameter×20 cm long) containing 1–2-d-old *T. japonicum* were placed in the refrigerator for a few minutes to make the parasitoids inactive. Inactive wasps were sexed under a stereomicroscope. Sexes of *Trichogramma* can be easily distinguished by their differences in antennal structure (Knutson, 1998, and personal observation). They were allowed to recover fully before being released into test arenas. Five mated adult females *T. japonicum* (1–2 d old) were released into the petri dish before covering with a lid. After 1 hr, the parasitoids were removed and the petri dishes were kept for observation. After 5–6 d, the treated eggs that appeared black in color (an indication of parasitization) on each egg card were recorded and counted by using a stereomicroscope. Control egg cards were also checked for parasitization.

Hydrocarbon compounds occur in almost all YSB-related material extracts. They are the most abundant compounds. Therefore, experiments were conducted to test whether these compounds actually account for the activity of the extraction/fraction in which they were found. The effect of about 10 synthetic hydrocarbon compounds on the parasitization rate was studied by using the above-described method of dual and multiple choice tests. For each treatment, 12 observations were made per day, and all treatments were conducted simultaneously on the same day. Experiments were repeated on three different days to avoid day-to-day variation. The mean percentage parasitization was calculated from 36 replicates.

Differences in the parasitization rate of treatments and control host eggs in multiple choice tests were statistically analyzed by one-way ANOVA (Origin version 7.5 software) followed by Scheffe's tests. In dual choice tests, the differences between two treatments were analyzed by paired *t*-test (Origin version 7.5).

Egg Samples

Trichogramma females oviposit readily in uncovered egg clusters of *Chilo* species. Eggs of *Scripophaga* species covered with hairs are obviously less accessible for a tiny parasitoid (van Vreden and Ahmadzabidi, 1986). The parasitoids' responses to treated YSB eggs and eggs of the artificial host, *C. cephalonioca*, were compared. As the results obtained with both host species were similar (±2% variation), eggs of *C. cephalonica* were used throughout the experiments because they were available abundantly at all times of the year in contrast to YSB eggs.

Behavioral Bioassays

Studies on the response of parasitoids to the chemicals present in crude extracts and also to the synthetic hydrocarbon compounds were conducted in the laboratory as follows: Behavioral observations were conducted in small flat-bottomed culture tubes (9 cm long; 2.5 cm diameter). Sample material (20 µl) was applied with a micropipette to a localized spot on a piece of absorbent paper (4×0.7 cm). After evaporation of solvent for about 2 min, paper strips were placed onto the inner side of the lid of the tube. For each treatment, controls were maintained in a separate tube with the same amount of hexane. One single adult-mated female was released into the glass tube before closing the lid. Observations on landing behavior, time spent on each treated and control patch, probing, and antennation were recorded visually. Landing and antennation of the treated spot by the parasitoid was denoted a positive response and assigned a highest activity. The observations took 20 min.

Behaviors were classified 0–4, with 0 as no reaction, no movement; 1 as moved upwards towards the paper strip; 2 as made circular movements around the paper strip; 3 as entered/landed on the paper strip; and 4 as antennated and probed on the chemical spot.

Parasitoids almost always responded quickly, either positively or negatively, to a treatment. Twenty females were tested for each treatment, and controls were tested at the same time. All treatments were repeated on three different days, and the total number of insects (replicates) for each treatment was 60. Almost all parasitoids showed a response to a treatment, so hardly any nonresponders were observed.

All experiments and observations were conducted in the laboratory at room temperature (28±2°C) and 65±5% RH, under a light bank with fluorescent lighting.

Results

Testing Effects on Parasitization Rate

Cuticular extracts of adult YSB bodies applied onto host eggs significantly affected parasitization by *T. japonicum*. More treated eggs were parasitized than controls (~91.5±0.5% vs. 8.25±0.33%, N=36) both in dual and multiple choice tests. Extracts of YSB larval frass also had positive effects on the parasitization rate in dual choice tests. More eggs treated with extracts of YSB larval frass were parasitized than controls. However, the effect was not as pronounced as with the adult cuticular extract. Host eggs treated with cuticular

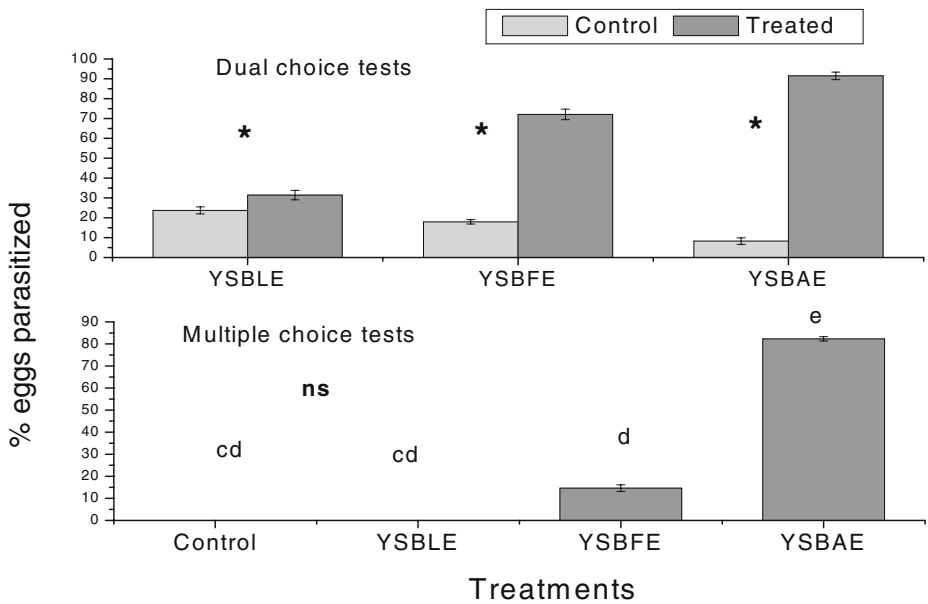


Fig. 1 Parasitization of differently treated host eggs by *T. japonicum* in dual and multiple choice tests. Percent eggs parasitized (means ± SE). Dual-choice test: significant differences (P<0.05) are indicated by *asterisks* (paired *t*-test). Multiple-choice tests: different *letters* indicate significant differences (ANOVA, P< 0.05, Scheffe's mean separation test), (N=50/each replicate, 36 replicates per treatment). *YSBAE* yellow stem borer adult cuticular extract, *YSBLE* yellow stem borer larval extract, *YSBFE* yellow stem borer larval frass

Table 1 Differences in parasitization rates of *T. japonicum* in host eggs treated with various fractions of YSB materials obtained by silica gel chromatography using different ratios of ethyl acetate in hexane

Chromatographic Fractions	Percentage of Host Eggs Parasitized (Mean ± SE)		
EA–HX	AE	FE	LE
2:98	6.5±3.2	3.5±1.1	0
4:96	20.4±5.4	5.2±2.2	2.4±1.3
6:94	7.6±2.2	27.8±3.1	1.9±1.4
8:92	68.4±2.8	8.4±2.2	0
10:90	6.2±2.2	5.6±3.4	0
12:88	78.1±3.1	10.2±3.9	0
20:80	19.5±4.2	17.1±2.7	0

Means ± SE are given of 36 replicates (N=50/each replicate)

AE adult cuticular extracts, *FE* extracts of larval frass, *LE* larval body extracts, *EA* ethyl acetate, *HX* hexane

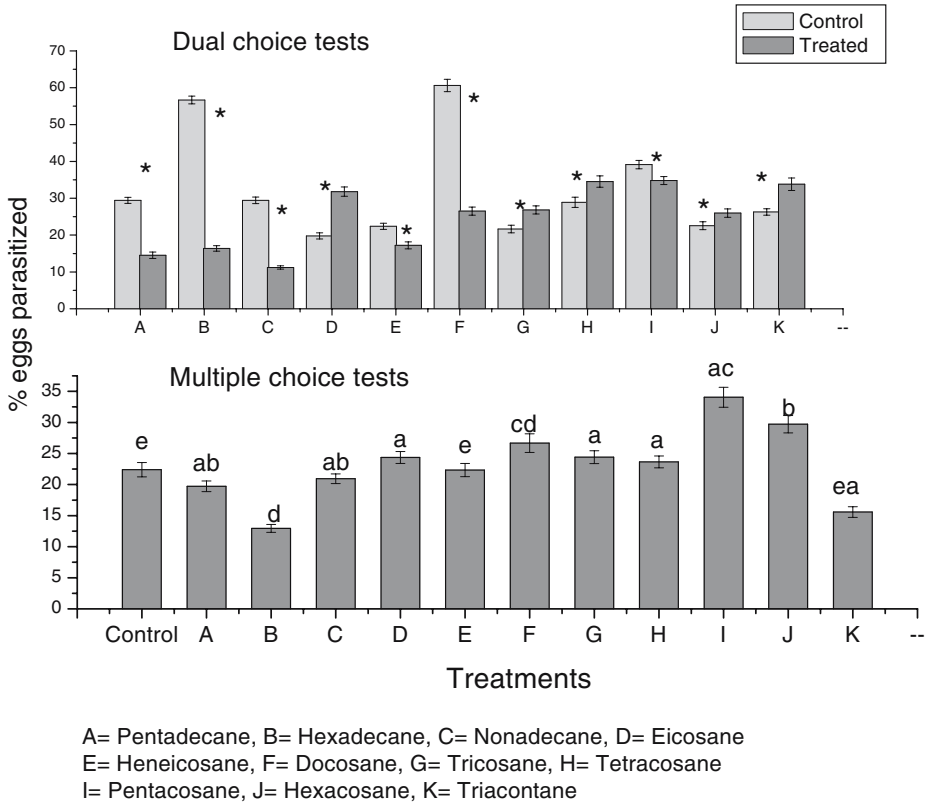


Fig. 2 Parasitization of eggs treated with synthetic hydrocarbons in dual-choice/multiple-choice tests by *T. japonicum*. Percent eggs parasitized (means ± SE). Dual-choice test: significant differences (P<0.05) are indicated by *asterisks* (paired *t*-test). Multiple-choice tests: different *letters* indicate significant differences (ANOVA, P<0.05, Scheffe's mean separation test), (N=50/each replicate, 36 replicates per treatment)

extracts of YSB larval bodies had the lowest effect on parasitization rates in dual choice tests, whereas no effect was detectable in multiple choice tests. The parasitoids response towards the adult cuticular extracts was significantly greater than to frass (~72.08±0.43%) or the larval extract (31.44±0.38%) (Fig. 1).

Among the chromatographic fractions, the eluate of the 12% ethyl acetate in hexane fraction of the adult extract was the most active one, followed by 8% ethyl acetate in hexane (Table 1). All other fractions were inactive. In the case of larval extracts, only the fractions eluted with 4 and 6% ethyl acetate in hexane stimulated a low percentage of oviposition. The remaining fractions were ineffective in causing any parasitization, and in these cases parasitization was significantly greater in solvent treated control eggs, suggesting an oviposition deterring activity of these fractions.

Treatment of the surface of host eggs with 20 μl of pentadecane, hexadecane, nonadecane, heneicosane, docosane, or pentacosane deterred oviposition. The percentage of eggs parasitized in these treatments was low compared to the other hydrocarbon compounds. Either these chemicals lack oviposition stimulatory activity or they possess deterrent activity (Fig. 2).

As was shown by the multiple choice tests, eicosane, docosane, tricosane, tetracosane, pentacosane, and hexacosane treatment of eggs enhanced the rate of parasitization (Fig. 2). Although host egg parasitization in heneicosane-, docosane-, and pentacosane-treated host eggs was less than the control in dual choice tests, no enhancement was found for heneicosane in the multiple choice test, and an enhanced parasitization was recorded for docosane and pentacosane during multiple choice bioassays. Similarly, triacontane was effective in stimulating the oviposition at a considerably higher rate in dual choice tests, but it was somewhat reduced in multiple-choice tests. The different results of dual- and multiple-choice tests might be due to the context (chemical background) in which a parasitoid (insect) experiences a chemical stimulus. Because this background was different in multiple- and dual-choice tests, the varying chemical context might explain these results (Vet and Groenewold, 1990; Mumm and Hilker, 2005; Faucher et al., 2006).

Table 2 Orientation responses of *T. japonicum* female to extracts of YSB adult, larval frass, and larval extracts

Samples	Score/Type of Response	Initial Time of Response (in min)
Crude extracts		
YSB AE	4	3
YSB FE	3	10
YSB LE	1	15
Hydrocarbons		
Pentadecane	1	0
Hexadecane	4	1
Nonadecane	4	3
Eicosane	0	0
Heneicosane	0	0
Docosane	4	0
Tricosane	4	15
Tetracosane	2	1
Pentacosane	4	1
Hexacosane	4	5
Triacontane	4	1

The type of response shown by 60 individual parasitoids is presented. 0=No response, 1=moved upwards, 2=landed on the paper strip, 3=moving around the patch, and 4=probing on the patch

AE adult cuticular extracts, *FE* extracts of larval frass, *LE* larval body extracts

Table 3 List of chemical compounds detected by GC–MS from the rice YSB adult cuticular extracts

1. Methyl cyclohexane
2. Ethyl cyclopentane
3. 1,2,3-Trimethyl cyclopentane
4. 2-Methyl heptane
5. 1,3-Dimethyl cyclohexane
6. 1,4-Dimethyl cyclohexane
7. Octane
8. 2,6-Dimethyl heptane
9. 1,1,3-Trimethyl cyclohexane
10. 2,3-Dimethyl heptane
11. 1-Ethyl-3-methyl cyclohexane
12. Nonane
13. Thiophene
14. Propyl cyclohexane
15. 2,6-Dimethyl octane
16. 3-Methyl nonane
17. 1,3,5-Trimethyl cyclohexane
18. Decane
19. Dodecane
20. Tetradecane
21. Hexadecane
22. Myristic acid
23. Octadecane
24. Phthalic acid
25. 6 Aza-5,7,12,14-tetrathiapentacene
26. Hexadecanoic acid
27. 3-Methyl nonadecane
28. Eicosane
29. Kaur-16-en
30. Octadecanoic acid
31. Docosane
32. Tetracosane
33. Pentacosane
34. Tricosane
35. Heptacosane
36. Squalene
37. Cholest-5-en-3-ol
38. Stigmasterol
39. Ethyl cyclohexane
40. C3 Benzene
41. Morphine
42. 3-methyl nonane

The total number of chemicals found was 61, but the chemicals present in less than 1% of the area were not included

Behavioral Bioassays

The bioassays on landing behavior in the small glass tube experiments revealed the wasps' responses towards the crude extracts, the chromatographic fractions, and the hydrocarbon compounds (Table 2). Parasitoids showed great preference for adult host cuticular extracts. In these treatments, wasps moved upwards and landed on the treated strip followed by ovipositor probing, which is an indication of the highest positive response. Extracts of larval frass also stimulated to a certain extent, although the parasitoids could only land on

Table 4 List of chemical compounds detected by GC–MS from the rice YSB larval frass

1. Methyl cyclohexane
2. Octane
3. Ethyl cyclohexane
4. Nonane
5. Decane
6. Dodecane
7. Tetradecane
8. Hexadecane
9. Octadecane
10. Phthalic acid
11. 3-Methyl heptadecane
12. Eicosane
13. 5-Eicosene
14. Nonadecane
15. Docosane
16. Heneicosane
17. Tetracosane
18. Tricosane
19. Hexacosene
20. 3 (4-*N*,*N*-dimethylaminophenyl)
21. Squalene
22. 3-Methyl pentadecane
23. 3-*O*-(trifluoromethyl) morphine
24. Hexadecanoic acid
25. Cyclotetracosane

The total number of chemicals found was 31, but the chemicals present in less than 1% of the area were not included

the paper strip containing the treatment spot. Larval extracts also evoked slight positive movements in the parasitoids; they made upward movements in the glass tube but did not reach the treated area. However, when testing control solvent strips, all wasps remained at the bottom of the glass tube without attempting to move upwards. These responses of *Trichogramma* were clear, always showing the same response to each chemical or extract.

The parasitoids' responses towards the various chromatographic fractions of adult extract eluted with different percents of ethyl acetate in hexane were as follows: 4%, 6%, 8%, and 12% ethyl acetate fractions stimulated quick directional movements, and almost within 1 min after introducing the insects, they moved towards the treated spot, landed, and started probing the patch (type 4 response). They remained there until the end of the experimental period. Only 2% and 10% ethyl acetate fractions elicited a type 2 response, i.e., only landing on the paper strip. Similar responses were shown by the parasitoids to the 2% fraction of the larval cuticular extracts.

Among the synthetic hydrocarbons tested for orientation behavior, hexadecane, docosane, tetracosane, pentacosane, and triacontane elicited maximum attraction reaching the target patch within or less than 1 min. This was followed by nonadecane, hexacosane, and tricosane. Interestingly, hexadecane and nonadecane, which failed to enhance parasitization, triggered an excellent orientation in this bioassay. Parasitoids were seen staying on these treatments even after the observation period (Table 2).

Chemical analysis of the active fractions by GC indicated the presence of different groups of chemicals. Among those found in chromatographic fractions of different crude extracts, hydrocarbons are the most abundant and prominent. The adult cuticular extracts of YSB contained most components compared to those present in larval frass (Tables 3 and 4) and (Figs. 3a–c). There were 52 hydrocarbons, 6 acids, 1 alcohol, 1 ester, and 1 terpene in

the cuticular extracts of the adult moths, whereas larval frass had 28 hydrocarbons, 2 acids, and 1 ester. However, a lower number of hydrocarbons were found in larval surface washings, i.e., 27 hydrocarbons, 1 acid. The active fraction (12% ethyl acetate) from the adult extract contained mainly saturated long chain alkanes and alkenes with carbon numbers ranging from C_{20} to C_{32}. The GC analysis of the extracts from YSB larval body

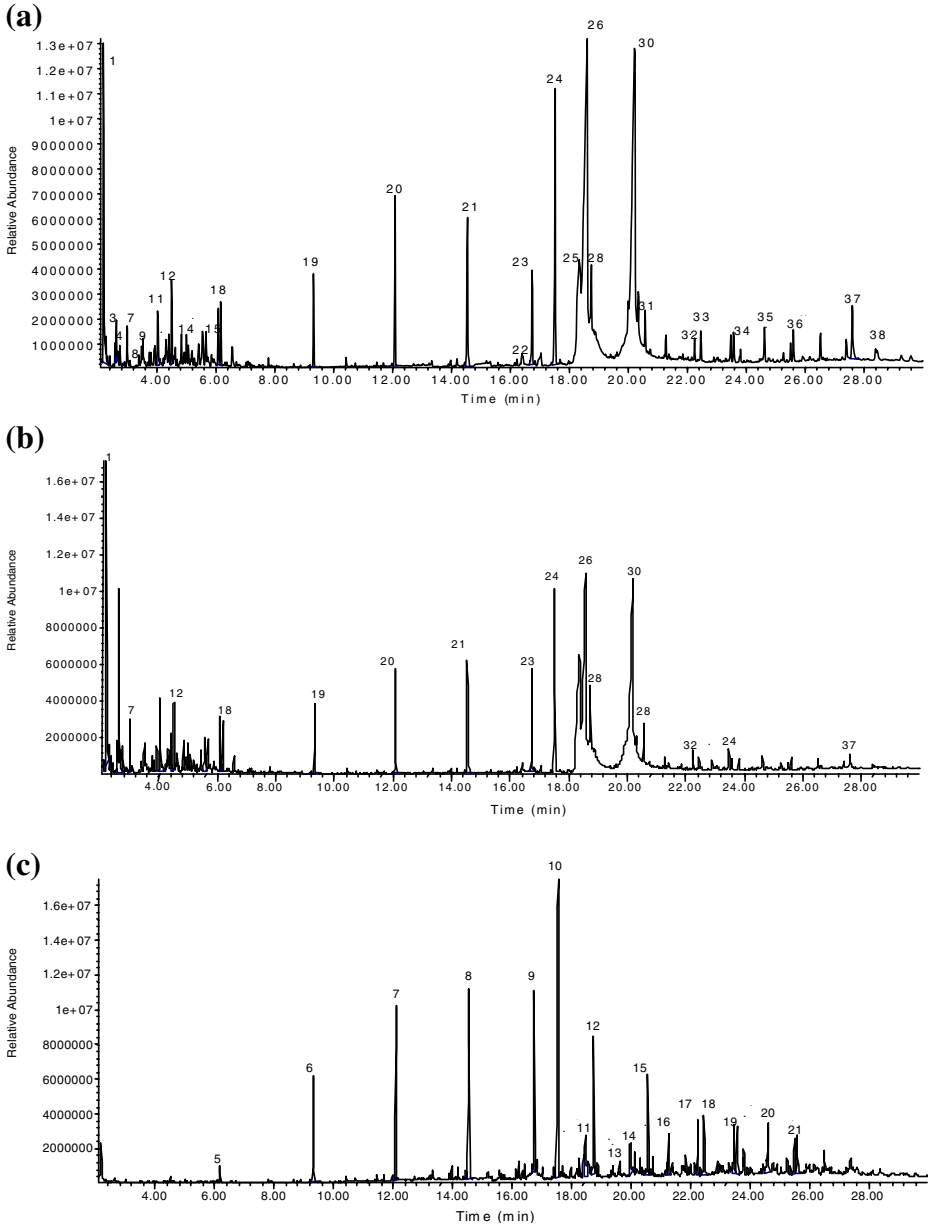


Fig. 3 Representative total ion current chromatograms of GC–MS analysis of adult cuticular extracts, the fraction eluted with 8:92 ethyl acetate in hexane (a), 12: 88 ethyl acetate in hexane (b), and larval frass (c) of YSB, *S. incertulas*. Compare numbers of (a) and (b) to Table 3 and numbers of (c) to Table 4

surface showed a different blend of chemicals, mostly those absent in the other two extracts (larval frass and adult). Toluene and 1,3-dimethyl benzene were present in larger quantities in larval YSB cuticular extracts than in the extracts of frass or the adult. All the fatty acids found in adult cuticular or frass extracts, such as palmitic, myristic, and oleic acids, as well as certain hydrocarbons, namely, tricosane and tetracosane, were totally absent from the larval body extracts. Squalene (2,6,10,14,18,22 tetracosahexaen) was present in all three extracts.

Discussion

The results demonstrate that cuticular extracts of adult host moths increase parasitization efficiency of *T. japonicum*. The active materials are hexane soluble. They indicate that the chemicals extracted from the surface of adult YSB cause arrestment of parasitoids in host vicinity and stimulate oviposition by *T. japonicum*. The parasitoid's stronger response to adult host cuticular extracts in comparison to larval frass or larval body extracts indicates that this response is specific to the adult host and not a general response to insect cuticular chemicals. Our findings support those of several previous studies that have shown that moth body extracts affect parasitoid behavior (Lewis et al., 1975a, b; Elzen et al., 1984; Gross et al., 1984; Nordlund et al., 1984; Boo and Yang, 2000).

Extracts of adult host moths are mainly extracts of wing scales. Previous studies have shown that scales left by ovipositing host females aid in actual location of host eggs by parasitoids. Kairomones in moth scales probably act as sign stimuli, eliciting intensified searching behavior, rather than as a guiding cue attracting and directing the parasitoid to the host (Boo and Yang, 2000). Scale shedding by ovipositing moths is either incidental or can have an adaptive value. Moth scales present at oviposition sites signal the presence of host eggs on a larger area than the eggs alone. The major compounds identified as attractant and oviposition stimulants for egg parasitoids are hydrocarbons, which generally serve many functions in insects. They comprise a significant portion of the cuticular lipids that prevent desiccation, and are important in chemical communication (Nelson and Blomquist, 1995). Parasites have evolved the ability to utilize hydrocarbon cues produced by their hosts as primary cues in locating their hosts. Contact chemicals that are often secreted by a host and are present in the host frass or webbing have been found to be important for a number of parasitoids (Dicke et al., 1990). These compounds seem to elicit a fixed behavioral pattern exhibited by antennal searching of a contaminated area, followed by ovipositor probing in some parasitoids. Tricosane, pentacosane, and hexacosane were the hydrocarbons present exclusively in the adult body washings. They may be important cues for host location and ovipositional stimulation in *T. japonicum*. Wäckers and Lewis (1994) suggest that egg parasitoids recognize host eggs in close range by sight, while chemical signals play a secondary role in host searching. Although several parasitoid species rely on visual cues in the close vicinity of host eggs, it is intriguing from our results that parasitoids, when confined with host eggs in small areas such as petri dishes in lab bioassays, could discriminate between host eggs treated with different chemicals.

Certain chemicals, such as docosane, tricosane, tetracosane, and pentacosane were isolated and identified by Jones et al. (1973) from scales of adult *Heliothis zea* and were shown to increase parasitism by the parasitoid *Trichogramma* when applied to host eggs. All these hydrocarbons were also found in YSB adult extracts. Additionally, GC and GC–MS analyses indicated the presence of several organic acids in YSB adult cuticular extracts.

Hexadecanoic acid (palmitic acid) and octadecanoic acid (oleic acid) are the two compounds commonly occurring in both active fractions, i.e., 8% and 12% ethyl acetate in hexane. They are assumed to contribute to an increase of parasitism by *T. japonicum*. Because these two compounds have a high number of carbon atoms, they might act as contact stimulants, whereas the chemicals with less than 10 carbon atoms are more volatile and might attract parasitoids to the near vicinity of the host eggs.

The triterpene squalene and a diterpene kaur 16-en were present in all three extracts studied. Behavioral effects of squalene in interactions among other organisms and on third trophic levels have been reported by Dutton et al. (2002), who observed the triggering of ovipositional probing behavior of the parasitic wasp *Pholetesor bicolor*. We assume a similar function of this chemical in *T. japonicum*. A small percentage of parasitoids preferred the eggs treated with the fractions of larval cuticular extracts eluted with 4% and 6% ethyl acetate in hexane, and perhaps the presence of squalene might be responsible for this.

It has been argued that stem boring behavior of several herbivore species avoids the release of feeding damage cues and reduces their accessibility to natural enemies (Strong et al., 1984). The extracts of body washings of the larva or the solvent were found to have little effect on parasitization by *T. japonicum*. Larvae of YSB live inside the feeding tunnels of the rice stem and are not exposed to the parasitoids. Hence, it is unlikely that parasitoids use chemical cues emanating from the larval body surface as cues in locating the egg masses of YSB. The intense antennation and the enhanced parasitization of host eggs treated with YSB larval frass are interesting as larval frass also occurs in the tunnels made by feeding host larvae. However, the entrance hole of the tunnel was usually filled with frass pushed out of the tunnel by the feeding stem borer larvae. Hence, larval frass may serve as host locating cue to the wandering parasitoid.

This study showed that adult YSB moths have contact kairomonal activity associated with their bodies. Hexane extracts of moth scales, wings, and abdominal tips were a source of kairomones eliciting positive responses in *T. japonicum*. Presence of chemicals on crop plants could enhance host finding chances and, thus, the percent parasitism and reproduction by parasitoids. Compounds that attract or arrest parasitoids could be used in treating crops with kairomones to enhance the success of parasitism, thus reducing the need for harmful pesticides. This would meet the increasing need to use biological control of pest insects that damage plants grown in monocultures.

Acknowledgments We are grateful to the Department of Biotechnology, New Delhi, India, for research funding, and staff of the National Integrated Pest Management Center, Hyderabad, India, for the supply of Corcyra egg cards. We thank Dr. U. V. R. Vijay Saradhi of the Indian Institute of Chemical Technology, Hyderabad, India, for helping in analytical work, N. Uday Lakshmi and P. Devanand for technical assistance, and Dr. Steven Foster, North Dakota University, USA, and Dr. Ilkka Havukkala, Genesis, New Zealand, for valuable suggestions.

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J Chem Ecol (2007) 33:75–84
DOI 10.1007/s10886-006-9211-z

Alkaloid Profiles, Concentration, and Pools in Velvet Lupine (*Lupinus leucophyllus*) Over the Growing Season

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Received: 14 July 2006 / Revised: 18 September 2006 / Accepted: 21 September 2006 /
Published online: 5 December 2006
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Abstract *Lupinus leucophyllus* is one of many lupine species known to contain toxic and/or teratogenic alkaloids that can cause congenital birth defects. The concentrations of total alkaloids and the individual major alkaloids were measured in three different years from different plant parts over the phenological development of the plant. All of the alkaloids were found in the different plant tissues throughout the growing season, although their levels varied in different tissues. Concentrations of total alkaloids and the individual alkaloids varied on an annual basis and in their distribution in the different tissues. Anagyrine levels were highest in the floral tissue, lupanine and unknown F accumulated to the greatest level in the vegetative tissue, and 5,6-dehydrolupanine accumulated to the highest level in the stem. These alkaloids appear to be in a metabolically active state with the teratogenic alkaloid anagyrine accumulating to its highest level in the developing seed. The latter is, thus, the phenological stage posing the greatest danger to grazing livestock.

Keywords Lupine · Anagyrine · Crooked calf

Introduction

Lupines (*Lupinus* spp.) are a common plant species on western U.S. rangelands. Several species contain alkaloids that can be toxic and/or teratogenic to livestock. Historically, lupines have been a major cause of sheep deaths from respiratory failure on foothill and mountain rangelands in the western US (Kingsbury, 1964). Ingestion of lupine by the dam was reported to cause congenital birth defects in calves (crooked calf disease) in the early 1960s (Binns et al., 1962; Shupe et al., 1967a). Additional research demonstrated that the principal time of insult was during the 40–70th day of gestation (Shupe et al., 1967b). The quinolizidine alkaloid, anagyrine (Keeler, 1976), and some piperidine alkaloids (Keeler and Panter, 1989; Panter et al., 1998) were shown to reduce fetal movement during this critical

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period of gestation (Panter et al., 1990), causing the spine and limbs to develop in contracted or misaligned positions. Reports of calf losses due to crooked calf disease in several western states continue to be reported (Bagley and Panter, 1996; Panter et al., 1997; Warnock, 1997).

The objective of this study was to evaluate the toxicity of velvet lupine (*Lupinus leucophyllus*) over the growing season. Concentration of the teratogenic alkaloid anagyrine and total alkaloid concentration was measured in plant parts over the growing season, and profiles of the major alkaloids were determined.

Methods and Materials

Site Description

The study was conducted in conjunction with a grazing study (Ralphs et al., 2006) in the channel scabland region of eastern Washington state, USA about 25 km southeast of Ritzville (N 46°15.347′, W 118°13.031′), where losses of crooked calves have been catastrophic. In the spring of 1997, over 650 calves on 13 ranches were born deformed and had to be destroyed; occurrence on some individual ranches was as high as 100%. Additional calves that were deformed but functional were sold at minimal prices (Clive Gay, unpublished data).

The study site was characterized by basalt plateaus with depressions and valleys carved by geologic floods from the ancient lake Missoula. The original plant community of the bottomland site was dominated by basin wildrye (*Leymus cinereus*), and on the slopes by Palouse prairie species, such as bluebunch wheatgrass (*Pseudoroegneria spicata*), and Idaho fescue (*Festuca idahoensis*). The site has been degraded by fire and overgrazing to the point that it is dominated by annual species such as cheatgrass (*Bromus tectorum*), fiddleneck (*Amsinckia intermedia*), tansy mustard (*Descurainia pinnata*), prickly lettuce (*Lactuca serriola*), and black mustard (*Brassica nigra*). Velvet lupine (*L. leucophyllus*) was one of the few perennial forbs remaining.

Lupine samples were collected at roughly 2-wk intervals during the growing season in 2001, 2002, and 2003. Ten uniform plants were harvested during each collection period throughout the length of the pasture at approximately noon. They were separated into parts (leaf, stem, flower, pod, and seed), dried at 60°C for 48 hr, and ground through a cyclone mill, and 100 mg of the dried plant material was weighed into a 16-ml screw top glass vial. Alkaloids were isolated following standard acid/base extraction procedures with 1% hydrochloric acid, chloroform, and dilute ammonium hydroxide, and quantified by a gas chromatography (GC) (Gardner and Panter, 1993). Deviations from the previously described procedure included the use of caffeine as an internal standard, and concentrations of alkaloids were quantified against a six-point anagyrine standard.

Alkaloid concentrations were measured in plant parts, and profiles of the major alkaloids are presented over the growth stages of the plant. In addition, alkaloid pools, or absolute amount of alkaloid, was calculated by multiplying the concentration by the dry weight of the plant part. Pools are more useful in interpreting ontogenetic and environmental changes in synthesis and distribution of secondary compounds in plants (Koricheva, 1999). Plants produce and allocate quantities of compounds, whereas concentrations reflect the distribution of these quantities in plant tissues, and are therefore, functions of plant biomass.

Statistical Analysis

Concentration of the teratogenic alkaloid, anagyrine, and total alkaloids, were compared among plant parts and over years by a mixed model analysis of variance (SAS, 1999). Year and plant part were the fixed effects, and individual plants were the random effect. There was a year by plant part interaction for both anagyrine and total alkaloids; thus, the model was reduced, and each part was analyzed separately across years. Means were separated by the PDIFF function within the mixed model, comparing all possible combinations.

The concentration of anagyrine in plant parts was graphed over the growing seasons each year to express their phenological development. Anagyrine pools and standard errors for floral parts and leaf/stem were also graphed over the growing season to illustrate the total amount of the teratogenic alkaloid in the plant over the season and between years. Profiles of the six dominant alkaloids in this population of velvet lupine are also presented by plant part and over the growing season.

Results

The GC alkaloid profile of velvet lupine shows the presence of six major alkaloids and a number of minor alkaloids (Fig. 1). Three of the major alkaloids in velvet lupine were determined by GC retention time and mass spectrometric data to be 5,6-dehydrolupanine, lupanine, and anagyrine (Kinghorn and Balandrin, 1983; Wink et al., 1995). The alkaloids F (mw=246), T (mw=262), and N (mw=278) do not appear to correspond to any known alkaloids. We are in the process of separating and isolating enough of these compounds for structural determination.

Concentration of anagyrine and total alkaloids differed among years ($P<0.003$), plant parts ($P<0.001$), and there was a year × part interaction ($P<0.001$). Anagyrine con-

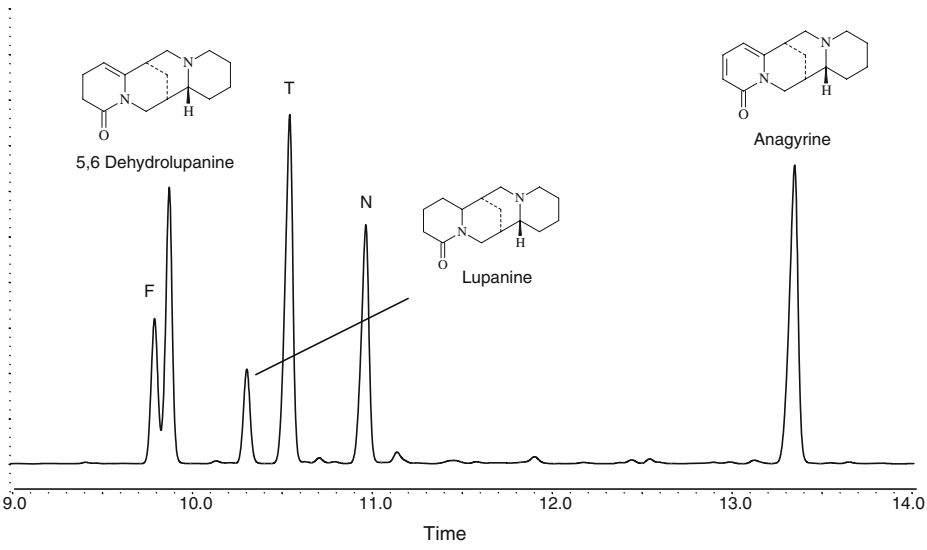


Fig. 1 Representative gas chromatogram showing quinolizidine alkaloids in velvet lupine

centration was greater in the floral heads than in the leaf and stem in each of the 3 yr (Table 1). Furthermore, the concentration of anagyrine in the floral head was greater in 2002 than the other 2 yr. Concentration of the total alkaloids was similar in the floral heads and leaves, but lower in the stem (Table 1). In addition, the total alkaloid content and the anagyrine content are not directly related in the different plant parts on a yearly basis. For example, the anagyrine concentration in floral heads was significantly higher in 2002 than in 2003, whereas the total alkaloid was similar in these 2 yr.

Anagyrine concentrations were greater in the floral parts than in the leaf and the stem throughout most of the growing season in all 3 yr (Fig. 2). The anagyrine concentration in the seed was two to three times greater than in the flower or pod. Anagyrine concentrations increased in the floral tissue until seed maturation and shatter, whereas its concentration in vegetative tissue remained constant or slightly decreased. After the seeds shattered from the pod around the first of July each year, anagyrine concentrations declined, suggesting that much of the anagyrine was contained in the seeds and/or catabolized as the seeds matured and shattered.

Anagyrine pools were calculated by multiplying the mass or weight of the plant part by the anagyrine concentration in the part. Anagyrine pools in the floral parts were greater than in the leaf and the stem through much of the growing season (Fig. 3). This results from the higher concentration of anagyrine in these parts and the increasing proportion of the plant's mass as the pods mature. Anagyrine pools in the floral parts steadily increased up to the point of seed ripening, which was subsequently followed by a significant decline after the seeds shattered from the pod. Anagyrine pools in the vegetative tissue remained constant or decreased.

Other Major Alkaloid Profiles

All of the other alkaloids were present in both floral, leaf, and stem tissue at some point within the growing season. Unknown F, 5,6-dehydrolupanine, and lupanine were present at higher levels in the leaves than in the floral parts (Fig. 4). Levels of these three alkaloids increased or stayed approximately the same from early growth to the point of seed maturity followed by a subsequent decline. Unknowns N and T were present in both the leaves and floral parts equally. They also increased in concentration as the plant matured, followed by

Table 1 Anagyrine and total alkaloid concentration of plant parts over years

Year	Anagyrine				Total Alkaloids			
	Floral Heads	Leaf	Stem	Mean	Floral Heads	Leaf	Stem	Mean
Percent Dry Weight (%)								
2001	0.36 (*b*)	0.11 (*a*)	0.10 (*b*)	0.19 (*B*)	0.98 (*B*)	1.19 (*a*)	0.41 (*b*)	0.86 (*B*)
2002	0.48 (*a*)	0.10 (*a*)	0.11 (*ab*)	0.23 (*A*)	1.31 (*a*)	0.96 (*b*)	0.52 (*b*)	0.93 (*B*)
2003	0.39 (*b*)	0.12 (*a*)	0.14 (*a*)	0.22 (*A*)	1.32 (*a*)	1.32 (*a*)	0.71 (*a*)	1.11 (*A*)
Mean	0.40 (*C*)	0.11 (*D*)	0.12 (*D*)		1.12 (*C*)	1.16 (*C*)	0.55 (*D*)	

(*ab*) Difference of individual plant parts between each year ($p<0.05$), (*AB*) mean of plant parts (Floral Heads, Leaf, Stem) between each year, (*CD*) mean of years (2001, 2002, 2003) between each plant part ($p<0.05$)

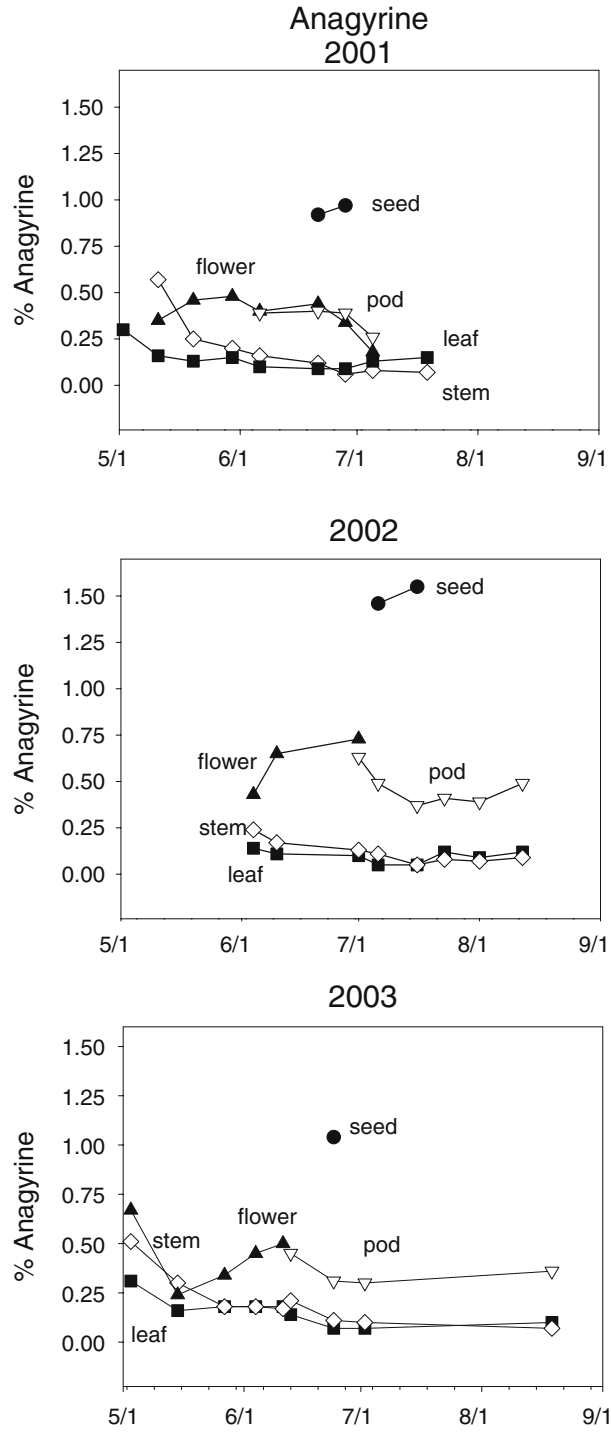


Fig. 2 Anagyrine concentration in plant parts over the growing season

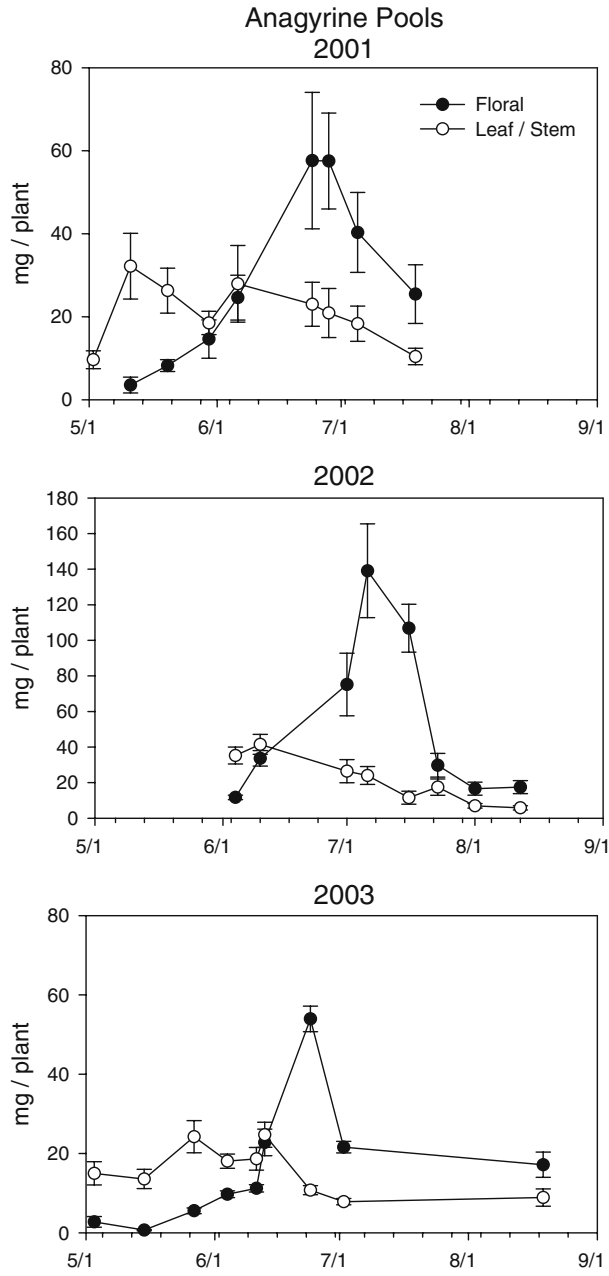


Fig. 3 Anagyrine pools in floral heads and leaf/stem over the growing season

a decline. Interestingly, anagyrine was the only alkaloid that showed greater levels in the floral parts than the other tissue parts.

In the stem, 5,6-dehydrolupanine is the dominant alkaloid (Fig. 5). Its concentration was two to three times greater than the other alkaloids in the stem during rapid growth and up to pod formation.

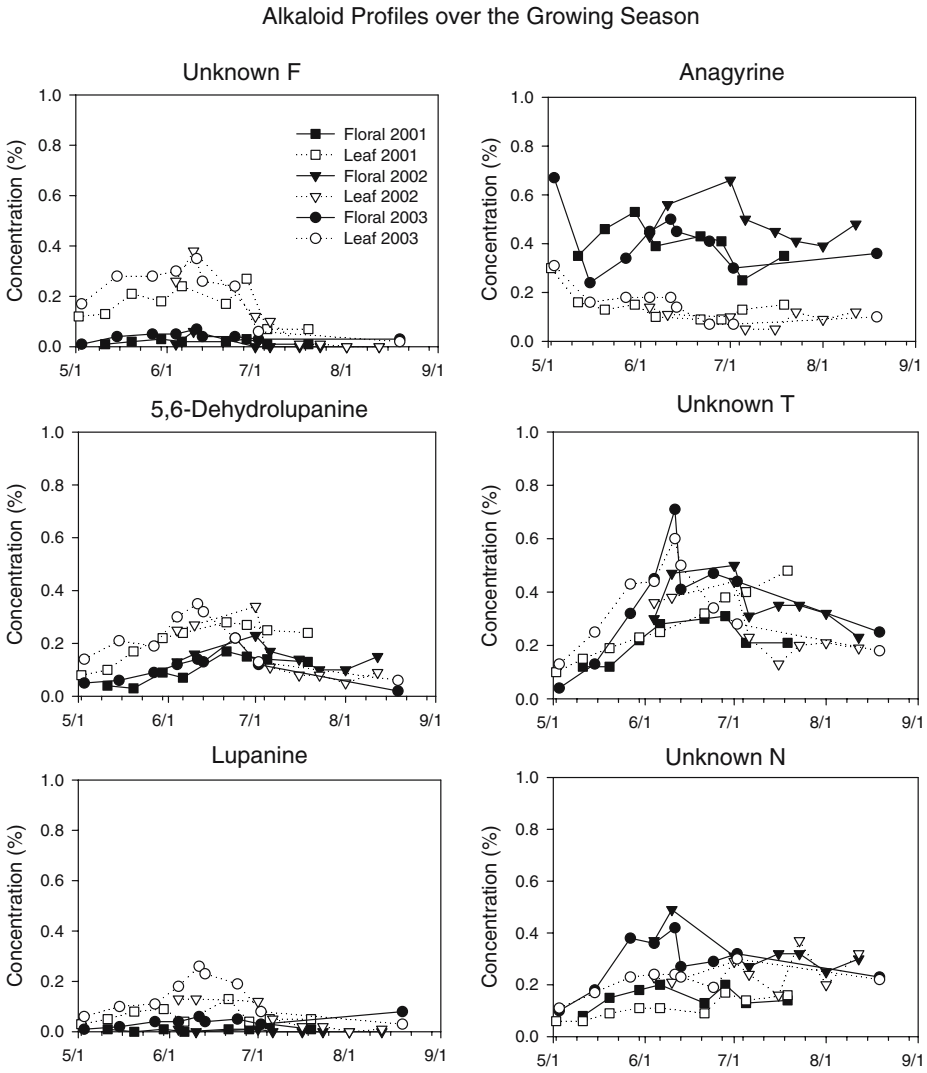


Fig. 4 Major alkaloids in floral parts and leaves over the growing season

Discussion

Velvet lupine contains a number of alkaloids that can be toxic and/or teratogenic to livestock. The concentrations of six alkaloids were evaluated throughout the growing season in different plant parts. Total alkaloids and anagyrine varied from year to year and in the different plant parts.

The teratogenic alkaloid anagyrine accumulates in the floral parts and reaches its highest concentration in the seeds. These results are similar to observations reported by Keeler (1976) in *L. caudatus* from different phenological growth stages. Furthermore, the anagyrine pools suggest that the majority of this toxic alkaloid is located in the floral parts. This is in contrast to lupanine and unknown F that accumulate to greater levels in

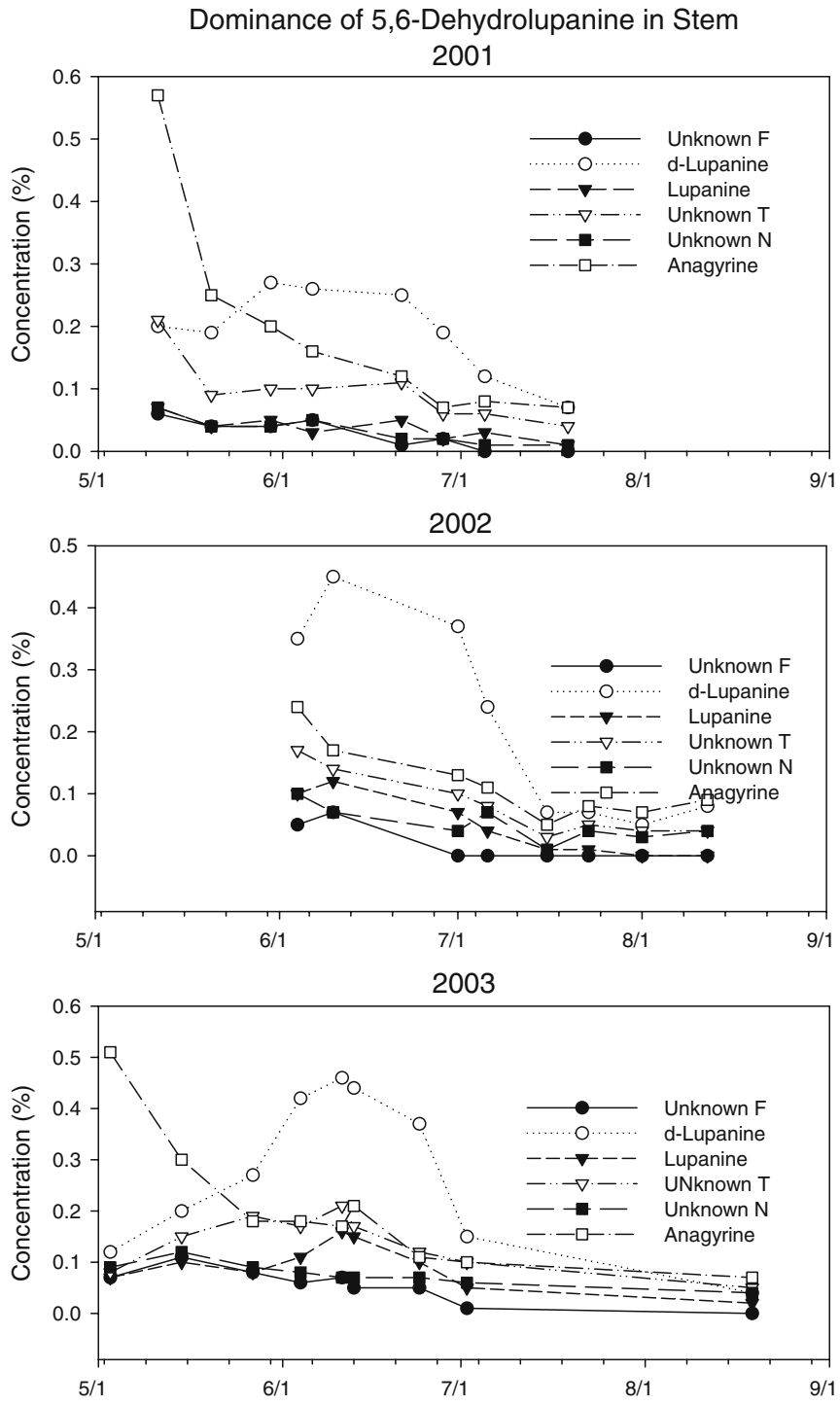


Fig. 5 Dominance of 5,6-dehydrolupanine (*d*-lupanine) in stems during rapid growth

leaves than the floral parts. In addition, 5,6-dehydrolupanine is the major alkaloid in the stem. The dominance of 5,6-dehydrolupanine in the stem suggests a transport function. Interestingly, it has been shown to be an intermediate metabolite in the biosynthesis pathways from lupanine to anagyrine (Cho and Martin, 1971). These results suggest that these compounds are in a metabolically dynamic state throughout the plant.

Wink and Witte (1984) reported that quinolizidine alkaloids in the annual lupine (*L. albus*) are synthesized in the chloroplast in leaves during the day, translocated through the phloem stream in the stem during the afternoon, are deposited in the pod and seeds, and decline during the night. This suggests that quinolizidine alkaloids are not waste products or end products of metabolism, but that they are metabolically dynamic compounds involved in defense from herbivory. In further support of this active metabolic process, it was observed that anagyrine in the seeds comprised only 13–38% of the total anagyrine pool in the floral head just before seed shatter. The results indicate that a significant portion of the pool of anagyrine remains in the empty pods and is then degraded and/or translocated from the middle of July to the first part of August. These results are consistent with a report from Wink and Hartmann (1981) that the concentration of quinolizidine alkaloids dropped 90% during senescence.

In summary, the quinolizidine alkaloids in lupine appear to be in a metabolically active state. The distribution of the individual alkaloids in the different tissues led us to speculate that unknown F and lupanine are synthesized in the leaf, transported in the phloem in the stem as 5,6-dehydrolupanine, and metabolized to anagyrine in the flowers, the pods, and the seeds. The teratogenic alkaloid anagyrine accumulates to its highest level in the floral parts and reaches its highest concentration in the seeds as they mature in late June. Anagyrine concentration drops dramatically as the seeds shatter in mid-July. The general breeding for cattle in the region where this study was performed is from May 1 to July (Ralphs et al., 2006). This puts the susceptible period of gestation to cause crooked calves (40–70 d of gestation) between approximately June 10 and October 8. Therefore, the high concentration of anagyrine in the mature seed overlaps the susceptible period of gestation, causing a high risk of crooked calf syndrome.

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J Chem Ecol (2007) 33:85–94
DOI 10.1007/s10886-006-9205-x

Cyanide in the Chemical Arsenal of Garlic Mustard, *Alliaria petiolata*

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Received: 3 July 2006 / Revised: 30 August 2006 / Accepted: 18 September 2006 /
Published online: 5 December 2006
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Abstract Cyanide production has been reported from over 2500 plant species, including some members of the Brassicaceae. We report that the important invasive plant, *Alliaria petiolata*, produces levels of cyanide in its tissues that can reach 100 ppm fresh weight (FW), a level considered toxic to many vertebrates. In a comparative study, levels of cyanide in leaves of young first-year plants were 25 times higher than in leaves of young *Arabidopsis thaliana* plants and over 150 times higher than in leaves of young *Brassica kaber*, *B. rapa*, and *B. napus*. In first-year plants, cyanide levels were highest in young leaves of seedlings and declined with leaf age on individual plants. Leaves of young plants infested with green peach aphids (*Myzus persicae*) produced just over half as much cyanide as leaves of healthy plants, suggesting that aphid feeding led to loss of cyanide from intact tissues before analysis, or that aphid feeding inhibited cyanide precursor production. In a developmental study, levels of cyanide in the youngest and oldest leaf of young garlic mustard plants were four times lower than in the youngest and oldest leaf of young *Sorghum sudanense* (cv. Cadan 97) plants, but cyanide levels did not decline in these leaves with plant age as in *S. sudanense*. Different populations of garlic mustard varied moderately in the constitutive and inducible expression of cyanide in leaves, but no populations studied were acyanogenic. Although cyanide production could result from breakdown products of glucosinolates, no cyanide was detected *in vitro* from decomposition of sinigrin, the major glucosinolate of garlic mustard. These studies indicate that cyanide produced from an as yet unidentified cyanogenic compound is a part of the battery of chemical defenses expressed by garlic mustard.

Keywords Allelopathy · *Alliaria petiolata* · Cyanide · Invasive plants · Plant defenses · *Sorghum sudanense*

Introduction

Cyanide is a well-known inhibitor of respiratory electron transport that is produced by over 2500 plant species including many food plants (Jones 1998). Although substantial debate

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exists on the role of cyanogenic compounds and cyanide in plants (Jones 1998; Gleadow and Woodrow, 2002), cyanide production has been invoked as an herbivore- and pathogen-resistance mechanism in many plants including clover, black cherry, and sorghum (Lindroth et al., 2000; Busk and Møller, 2002; Fitzgerald et al., 2002; Gleadow and Woodrow, 2002). In addition, cyanogenic compounds in plants like cassava appear to have roles in storage and mobilization of nitrogen (Gleadow and Woodrow, 2002; Jorgensen et al., 2005). Cyanide production in each of the aforementioned species typically reaches levels that can impact animal or human health (Jones 1998). Cyanide production has been reported in some members of the Brassicaceae, including *Brassica oleraceae*, *B. juncea*, *B. rapa*, *B. napus*, and *Raphanus sativus* (Sexton and Howlett, 2000; Chandra et al., 2004), but levels found thus far in plants in this family have been minimal and generally well below those considered to affect plant or animal health. In turn, the source of the detectable cyanide in brassicaceous plants is unknown, although the decomposition of glucosinolates, a ubiquitous group of defensive secondary compounds in this family, is one candidate (Sexton and Howlett, 2000).

Garlic mustard *Alliaria petiolata* [(M. Bieb) Cavara and Grande; Brassicaceae] is a Eurasian biennial herb that is an important invasive plant in North America. This plant has several known or presumed impacts on the flora and fauna in its invaded range that are apparently chemically mediated. These include allelopathic interactions with plants (Prati and Bossdorf, 2004), mycorrhizal fungi (Roberts and Anderson, 2001; Stinson et al., 2006), and herbivorous insects (Porter 1994; Haribal and Renwick, 1998; Haribal et al., 2001). Garlic mustard is also grazed little by mammalian or avian herbivores (D. Cipollini, personal observation). Like all brassicaceous plants, garlic mustard produces glucosinolates and defense proteins in both a constitutive and inducible fashion (Vaughan and Berhow, 1999; Cipollini et al., 2005). Garlic mustard is also rich in flavone gycosides, including isovitexin 6″-*O*-β-D-glucopyranoside, and it produces a novel cyanoallyl glycoside, alliarinoside; both have demonstrable effects on insect resistance (Haribal and Renwick, 1998; Haribal et al., 2001; Cipollini et al., 2005). It is likely that this suite of defenses contributes to the invasive success of this plant in novel habitats.

In this paper, we report that garlic mustard produces substantial quantities of cyanide from its tissues, an as yet undescribed component of the chemical arsenal of this plant. We first examined cyanide production in leaves and roots of young plants from a single population of garlic mustard, and then compared levels found in shoots to those of several members of the Brassicaceae. Second, we compared temporal variation in cyanide production in the youngest and oldest leaves of garlic mustard to that of a known cyanide producer, *Sorghum sudanense* (Sudan grass). Third, we examined leaf age-specific cyanide production in garlic mustard in the presence and absence of aphid herbivory. Fourth, we examined variation among garlic mustard populations in constitutive and inducible cyanide production. Finally, we examined whether the decomposition of sinigrin, the primary glucosinolate in garlic mustard (Vaughan and Berhow, 1999), could be the source of cyanide produced by this plant.

Methods and Materials

Experiment 1: Demonstration of Cyanide in Roots and Shoots of Garlic Mustard Seeds of garlic mustard were collected from plants growing naturally in the Wright State University forest preserve, cold stratified, and grown in the greenhouse in Pro Mix BX potting

medium, as described by Cipollini et al. (2005). Most experiments were conducted with the Wright State population, although experiment 5 includes several other populations. In this experiment, four plants were grown for 3 wk at which time all stems, leaves, and petioles present on each plant were collected and pooled for cyanide analysis, as described below. Roots of each plant were carefully extracted from the soil at the same time and rinsed free of soil with distilled water before cyanide analysis. Cyanide production by above-ground tissues was compared to that of roots with one-way analysis of variance (ANOVA) on SAS (version 9.1). Means were compared using Tukey's test in this and all other experiments.

Experiment 2: Comparisons of Cyanide Production in Leaves of Garlic Mustard with Other Brassicaceous Plants Seedlings of *Arabidopsis thaliana* (ecotype Columbia), *Brassica kaber* (wild collected), *B. rapa* (wild collected), and *B. napus* (cv. Westar), were grown from seed as described above, but without cold stratification. Garlic mustard was grown from cold-stratified seed as described above. All leaf material present on five 3-wk-old plants of each species was collected and pooled for analysis of cyanide. Cyanide production was compared among species with one-way ANOVA.

Experiment 3: Temporal Variation in Cyanide Production in Leaves of Garlic Mustard in Comparison to Sudan Grass This experiment was designed to compare variation in cyanide production in garlic mustard to a known cyanide-producing plant. Garlic mustard and Sudan grass [*S. sudanense* (cv. Cadan 97)] were grown from seed individually in pots in Pro Mix BX, as described above. Starting 2 wk after germination, the youngest fully expanded leaf and the oldest fully expanded leaf were collected from five plants of each species for cyanide analysis. Harvests continued in this manner from a subset of previously unsampled plants once per week for the next 8 wk, although the oldest leaves on each plant could be sampled for only 4 wk until they were too senescent to sample. This collection scheme isolates the effect of plant age on cyanide production in leaves of two distinct developmental stages. Cyanide production through time was compared between garlic mustard and Sudan grass with repeated-measures ANOVA, with time as the within-subject effect and species as the between-subjects effect. As the youngest leaves on each plant were sampled for 8 wk, and the oldest for only 4 wk, cyanide production was statistically examined separately for the youngest and oldest leaves.

Experiment 4: Leaf-specific Cyanide Production in the Presence of Aphid Herbivory This experiment examined within-plant variation in cyanide production among leaves in garlic mustard, but the plants were impacted by an outbreak of green peach aphids (*Myzus persicae*) in our greenhouse. When plants grown as described above were 40-d-old, we classified subsets of them as noninfested or infested (~20–30 aphids per plant). Infested plants were affected by aphids for approximately 2 wk at the time they were sampled. Fully expanded third (old), fourth (intermediate), and fifth (young) true leaves of garlic mustard plants were collected from 10–13 plants in each aphid infestation group for analysis of cyanide. Cyanide production was compared among aphid exposures and leaf ages by using two-way ANOVA, with aphid exposure and leaf age nested within aphid exposures as effects.

Experiment 5: Population Variation in Constitutive and Salicylic Acid-induced Levels of Cyanide Seeds of five populations of garlic mustard collected in the wild from Dalsland, Sweden; Marmontweg and Elderveld, the Netherlands; Hanau, Germany; and Ligonier, Pennsylvania, were cold-stratified and grown as described above. When plants were 8 wk

old, five plants from each population were sprayed with a 0.05-M solution of salicylic acid, as described by Cipollini et al. (2004). Salicylic acid induces many defense-related genes in plants (Mauch-Mani and Metraux, 1998), but its effect on cyanide production has never been examined. Leaf material was collected from the fully expanded fourth true leaf of each of these plants along with each of five control plants. Cyanide production was compared among populations and salicylate treatments with two-way ANOVA.

Experiment 6: Assessment of Sinigrin as a Source of Cyanide In this experiment, we evaluated the ability of sinigrin, the primary glucosinolate in garlic mustard (Vaughn and Berhow, 1999), to liberate cyanide upon enzymatic decomposition with thioglucosidase. We incubated 0.1 ml of sinigrin (Sigma Chemical, St. Louis, MO, USA) at concentrations of 0.125, 0.25, 0.5, 1.0, and 2.0 mg/ml with 0.1 ml of 3 mg/ml thioglucosidase (E.C. 3.2.3.1 from *Sinapis alba*; Sigma Chemical) suspended in sodium phosphate buffer in 25 ml center-well incubation flasks, and followed the protocol for cyanide trapping and analysis described below.

Cyanide Analysis We analyzed endogenous cyanide production in our tissues using a method modified from Lambert et al. (1975). Briefly, a weighed amount of fresh plant material (~0.02–0.05 g depending on tissue and species) was ground in a cold mortar with 0.5 ml of ice-cold 0.1-M sodium phosphate buffer. The homogenate was quickly transferred to a 25-ml center-well incubation flask, and the mortar was rinsed into the flask with an additional 0.5 ml of phosphate buffer. To trap cyanide in the flask, 0.2 ml of 0.1 M NaOH was placed in the center well, and the flasks were sealed and incubated at room temperature for 18 hr. To quantify cyanide present in the trap solution, 0.1 ml of 1 M acetic acid were added to the 0.2-ml trap solution (which was diluted as needed), followed by 1 ml of 0.25% succinimide/0.025% *N*-chlorosuccinimide reagent and 0.2 ml of 3% barbituric acid–pyridine solution. After incubation at room temperature for 10 min, absorbance at 580 nm was recorded for each sample in a spectrophotometer. Cyanide levels were quantified relative to a standard curve made with potassium cyanide and are expressed as milligram cyanide per kilogram tissue fresh weight (=ppm FW).

Results

Experiment 1 Three-week-old garlic mustard plants produced a mean level of 44.4 (±2.17) ppm cyanide from their aboveground tissues (including stems, leaves, and petioles) and 5.4 (±2.13) ppm from their roots. These levels were significantly different from one another ($F_{1,\ 6}$=212.5, P<0.001).

Experiment 2 Leaf cyanide production varied among the five brassicaceous species examined ($F_{4,\ 14}$=526.0, P<0.001, Table 1). Levels of cyanide in leaves of 3-wk-old garlic mustard plants were 25 times higher than in leaves of 3-wk-old *A. thaliana* plants and over 150 times higher than in leaves of 3-wk-old *B. kaber*, *B. rapa*, and *B. napus* plants.

Experiment 3 Changes in cyanide production through time by the youngest fully expanded leaf varied between garlic mustard and Sudan grass (time × species: $F_{7,\ 2}$=54.02, P=0.018, Fig. 1). Cyanide production in the youngest leaf of Sudan grass was five times higher than

Table 1 Mean (±1 SE) cyanide levels in leaves of 3-wk-old seedlings of five brassicaceous species

Species	Cyanide (ppm)
Alliaria petiolata	108.9 (7.82)[a]
Arabidopsis thaliana	4.19 (2.83)[b]
Brassica kaber	0.733 (0.571)[c]
Brassica rapa	0.617 (0.562)[c]
Brassica napus	0.433 (0.096)[c]

Means followed by different letters are significantly different at $P=0.05$.

in garlic mustard on the first sampling date, and it steadily declined to minimal levels through the 8-wk-time course as plants aged. In contrast, cyanide production declined little through time in garlic mustard, and by the fifth week of the time course through the end, cyanide levels in the youngest leaves of garlic mustard plants were significantly higher than in Sudan grass. A similar pattern was observed in cyanide production in the oldest fully expanded leaf of garlic mustard and Sudan grass through the 4-wk-time course (time × species: $F_{3,\ 6}=150.54$, $P<0.001$, Fig. 2). The decline in cyanide production in the oldest leaves in Sudan grass through time was even more dramatic than in the youngest leaves.

Experiment 4 Cyanide production varied among aphid-infested and noninfested plants ($F_{1,\ 64}=25.56$, $P<0.001$) and among leaf ages within each aphid treatment ($F_{4,\ 64}=10.72$, $P<0.001$, Table 2). Cyanide production was higher in young leaves than in older leaves in general and was lower in aphid-infested plants across all leaf ages.

Experiment 5 Significant variation in constitutive and salicylic acid (SA)-inducible cyanide production in leaves of 8-wk-old garlic mustard plants was detected among populations (pop: $F_{4,\ 40}=4.21$, $P=0.006$; pop × SA: $F_{4,\ 40}=8.4$, $P<0.001$, Table 3). Constitutive cyanide production was higher in plants from Dalsland, Marmontweg, and Elderveld than

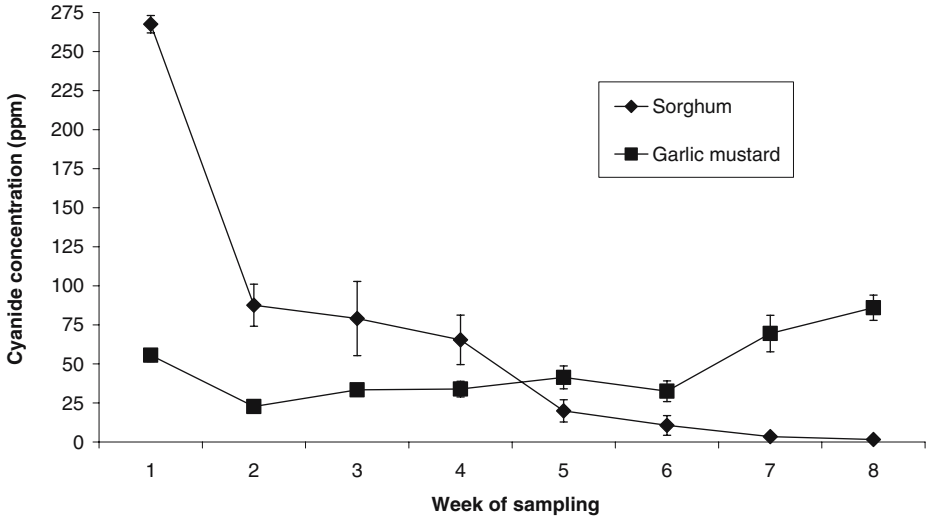


Fig. 1 Mean (±1 SE) cyanide levels in the youngest fully expanded leaf of garlic mustard and Sudan grass (*Sorghum sudanense*) through an 8-wk time course as plants aged. Plants were 2 wk old at the first sampling

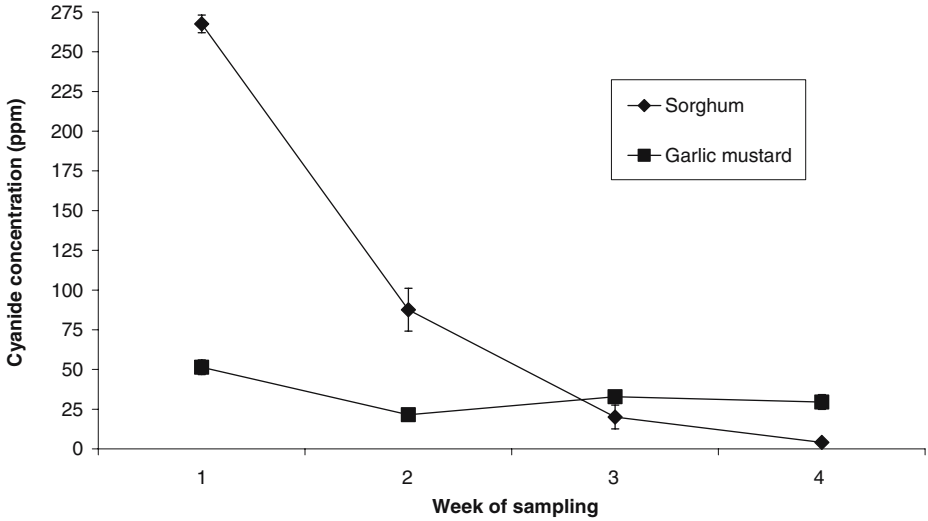


Fig. 2 Mean (±1 SE) cyanide levels in the oldest fully expanded leaf of garlic mustard and Sudan grass (*Sorghum sudanense*) through a 4-wk time course as plants aged. Plants were 2 wk old at the first sampling

in plants from Hanau and Ligonier. Although SA had little effect on cyanide production in plants from Dalsland, Marmontweg, and Ligonier, it strongly induced cyanide production in plants from Hanau and strongly reduced production in plants from Elderveld.

Experiment 6 Cyanide production was not detectable from the decomposition of sinigrin at any concentration in our experimental setup.

Discussion

Garlic mustard, an important invasive plant of forest understories throughout North America, produces a wealth of chemical defenses (e.g., Cipollini et al., 2005). We demonstrate for the first time here that garlic mustard produces substantial amounts of cyanide from its tissues.

Table 2 Mean (±1 SE) cyanide levels in young, intermediate, and old leaves of 5-wk-old seedlings of garlic mustard in the presence and absence of green peach aphids

Treatment	Leaf age	Cyanide (ppm)
Aphids absent	Young	66.2 (8.74)[a]
	Intermediate	38.7 (5.39)[b]
	Old	40.4 (5.00)[b]
Aphids present	Young	47.9 (6.68)[a]
	Intermediate	14.9 (2.13)[b]
	Old	16.9 (2.1)[b]

Means within an aphid treatment with different letters are significantly different at P=0.05.

Table 3 Mean (±1 SE) cyanide levels in leaves of 8-wk-old plants of five garlic mustard populations treated with 0.05 M salicylic acid or a control solution

Population	Salicylate Treatment	Cyanide (ppm)
Dalsland, Sweden	−	30.0 (4.2)[a]
	+	25.4 (4.3)
Elderveld, Netherlands	−	32.0 (4.6)[a]*
	+	11.4 (4.0)
Marmontweg, Netherlands	−	27.0 (4.7)[a]
	+	23.1 (4.1)
Hanau, Germany	−	14.4 (4.1)[b]*
	+	36.7 (4.3)
Ligonier, Pennsylvania	−	17.2 (4.0)[b]
	+	23.4 (4.6)

Means followed by different letters differ significantly at $P=0.05$ in the absence of salicylic acid. Means followed by asterisks indicate populations where salicylic acid significantly affected cyanide levels.

Cyanide contents reached by garlic mustard in some experiments were over 100 ppm FW in leaves, a level considered to be acutely toxic to humans (and other vertebrates) for plants like cassava (Jorgensen et al., 2005). In other experiments, levels in leaves ranged from 20 to 85 ppm, levels that are still considered to be moderately toxic (Jorgensen et al., 2005). As cyanide has known biological activity against animals, plants, and microbes (Jones 1998; Blenis et al., 2004; Gonzalez and Sotomayor, 2005), cyanide may contribute to some of the reported allelopathic effects of garlic mustard on other plants, insects, and mycorrhizal fungi (e.g., Porter 1994; Prati and Bossdorf, 2004; Stinson et al., 2006), and to its known deterrence of mammalian or avian herbivores (D. Cipollini, personal observation). It should be noted that we expressed cyanide content in garlic mustard per unit fresh weight, although many authors express cyanide content per unit dry weight (e.g., Lindroth et al., 2000). Expressing cyanide content in garlic mustard per unit dry weight would increase levels substantially, accounting for the average moisture content of leaves.

Within each plant, cyanide production was much higher in leaves than in roots and generally declined with leaf age. These findings are consistent with patterns of cyanide production in the foliage and roots of many other cyanogenic plants (e.g., Vickery et al., 1987; Busk and Møller, 2002; Fitzgerald et al., 2002; Jorgensen et al., 2005). In plants where the roots contain high levels of cyanide, like cassava, cyanide production by leaves is thought to be the source of cyanogenic compounds that are subsequently transported to roots (Jorgensen et al., 2005). Unlike *S. sudanense* studied here, cyanogenic potential in the youngest leaf of garlic mustard did not decline as plants aged. This indicates that garlic mustard consistently maintains the potential for cyanide toxicity in young leaves throughout much of its first season of growth, a pattern also seen in some other cyanogenic plants (e.g., Gleadow and Woodrow, 2000).

Interestingly, cyanide production was lower in aphid-infested plants across all leaf ages than in noninfested plants. Because we classified plants as infested or noninfested after the aphids had time to choose plants, it is possible that the patterns we observed resulted from aphid selection of the more weakly cyanogenic individuals in our experiment. However, as can be seen in the mean cyanide contents of the noninfested plants, individuals do not vary to a large extent in cyanide production in this population, which is known to be relatively genetically homogenous for other growth and defense characteristics (Cipollini 2002). Thus, it is more likely that aphid feeding somehow caused the differences in cyanide contents that we observed. For example, aphid feeding may have led to the volatilization of

cyanide from intact tissues before their analysis by enabling cyanide precursors and enzymes to mix *in planta*, thus lowering the cyanogenic potential of the tissue at the time of analysis. Alternatively, aphid feeding may have decreased cyanide precursor production either directly by downregulating gene expression or indirectly through nutrient limitation of precursor production. As cyanide production is increased by nitrogen fertilization in many plants (e.g., Busk and Møller, 2002; Jorgensen et al., 2005), aphid feeding may have reduced cyanide content by lowering tissue nitrogen content.

Populations varied to some extent in the constitutive and inducible production of cyanide, similar to other cyanogenic plant species (e.g., Lindroth et al., 2000; Schappert and Shore, 2000), but none of the six total populations studied here was acyanogenic. We have observed differences in defense production among populations of garlic mustard (Cipollini et al., 2005), but not enough populations were sampled in the current study to place our observations in an evolutionary or ecological context. Salicylic acid increased, decreased, or had no effect on cyanide production, depending upon the population studied. Salicylic acid is involved in the induction of numerous defenses in plants in response to biotic stimuli (Mauch-Mani and Metraux, 1998) and is known to induce cyanide-resistant respiration in plants (Kapulnik et al., 1992). Its effect on cyanide production has not been examined in any plant to our knowledge, but it has been shown to suppress the expression of dhurrinase mRNA in *Sorghum bicolor* (Salzman et al., 2005), which would presumably decrease cyanide production from the cyanogenic glycoside, dhurrin, found in these plants.

Garlic mustard produces substantially more cyanide than any brassicaceous plant studied to date (e.g., Sexton and Howlett, 2000; Chandra et al., 2004), including *A. thaliana* and the three *Brassica* species examined in this study. This appears to make garlic mustard unique among members of this family, which are characterized by their production of glucosinolates; cyanide production, however, has not been widely surveyed in this family. Only a few plant species are known as producers of both glucosinolates and cyanogenic glycosides, including *Carica papaya* in the Caricaceae family (Bennett et al., 1997), which is a member of the Capparales order along with members of the Brassicaceae family (Rodman et al., 1998). It has been suggested that glucosinolate production arose in an already cyanogenic species (Rodman et al., 1998; Halkier and Gershenzon, 2006), and it is perhaps surprising that so few members of the Brassicaceae have been confirmed to be cyanogenic.

Regardless of origin, cyanide production by garlic mustard may contribute to its unpalatability toward mustard specialist herbivores that typically prefer glucosinolate-containing plants (Porter 1994; Haribal and Renwick, 1998; Haribal et al., 2001) and some of its other allelopathic effects. We have noted anecdotally that treatment of garlic mustard leaves by freezing followed by subsequent thawing and incubation at room temperature makes them substantially more palatable and fosters better growth of larvae of the cabbage looper, *Trichoplusia ni* (D. Cipollini, unpublished data). This effect may be explained, in part, by the freezing-induced loss of cyanide from tissues. Interestingly, garlic mustard is also distinguished among members of its family by a characteristic garlic odor when its leaves are crushed (thus its common name). To our knowledge, the chemical profile and significance of this volatile blend has not been examined, but cyanide is certainly a part of it.

The source of cyanide in garlic mustard could not be determined in this study. The decomposition of glucosinolates has been suggested as a possible source in brassicaceous plants, but this does not appear to be the source in garlic mustard. Garlic mustard contains sinigrin (2-propenyl glucosinolate) as its primary glucosinolate (Vaughan and Berhow, 1999), but neither we nor Sexton and Howlett (2000) found that the thioglucosidase-

catalyzed hydrolysis of this compound could liberate cyanide *in vitro*. Moreover, the *Brassica* species and *A. thaliana* studied here are each well known as producers of glucosinolates (e.g., Cipollini et al., 2003, 2004; Chandra et al., 2004), yet each gave rise to negligible levels of cyanide in this study. The conversion of 1-amino-cyclopropane 1-carboxylic acid (ACC) into ethylene results in some cyanide production in plants, but levels seen here are much higher than those noted to be produced in this manner in other plant tissues (Yip and Yang, 1998). Although not initially considered a cyanogenic compound, garlic mustard contains a novel cyanoallyl glycoside, alliarinoside, with a –CN group as part of the molecule (Haribal et al., 2001). It is possible that either the enzymatic or nonenzymatic decomposition of this compound could yield volatile cyanide. If this compound is not the source of cyanide, then either other glucosinolates or an as yet undescribed cyanogenic compound may be the source.

Acknowledgments We thank Stephanie Enright for technical assistance, the Ohio Board of Regents for funding, and a simple curiosity that motivated this project. Martin Heil, Nicole van Dam, and Harriet Hillstrom kindly provided seeds of European garlic mustard plants. Two anonymous reviewers substantially improved the manuscript.

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J Chem Ecol (2007) 33:95–103
DOI 10.1007/s10886-006-9215-8

Interactive Effects of Leaf Damage, Light Intensity and Support Availability on Chemical Defenses and Morphology of a Twining Vine

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Received: 23 July 2006 / Revised: 2 October 2006 / Accepted: 5 October 2006 /
Published online: 17 November 2006
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Abstract In a greenhouse study, we evaluated the effect of the light environment and support availability on the induction of tropane alkaloids (TAs) after leaf damage in the twining vine *Convolvulus arvensis*. We also tested whether leaf damage modifies the phenotypic responses of the plant to shade and physical support. We found a consistent pattern of induction of TAs after leaf damage in each environmental condition. The induction of TAs was differentially affected by combinations of support and light treatments. In the sun, prostrate and climbing vines exhibited similar induced responses. In the shade, prostrate vines showed greater induced responses. Thus, vines showed the greatest chemical induction when damage occurred in a resource-poor environment (shade), and there was no cue (support) of future increase in resource uptake. Damaged plants showed reduced plasticity to shading in leaf shape and internodes and petiole length in comparison with control, undamaged plants. Herbivory and/or induced responses to herbivory may limit adaptive plant responses to the environment. Therefore, the negative consequences of herbivory on plant fitness might be magnified in a context of changing environments.

Keywords Climbing plants · Herbivory · Induced responses · Physical support · Shade

Introduction

Induced responses to herbivory in plants have been amply reported (Karban and Baldwin, 1997; Zangerl, 2003; Agrawal, 2005) and may be affected by environmental conditions (Gianoli and Niemeyer, 1996; Cipollini and Bergelson, 2001; Costa-Arbulú et al., 2001). The interplay between biotic and abiotic factors may thus determine plant phenotypic responses (Izaguirre et al., 2003, 2006; Cipollini, 2004). In the case of climbing plants, light intensity and support availability are environmental factors of fundamental importance

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(Putz, 1984; Gentry, 1991; Ray, 1992). In this regard, forest lianas and herbaceous vines exhibit both morphological specializations and adaptive behaviors that allow a better exploitation of solar radiation (Darwin, 1875; Peñalosa, 1983; Lee, 1988; Putz and Mooney, 1991). There is evidence of induced responses to herbivory in climbing plants (Rausher et al., 1993; Schierenbeck et al., 1994; Fordyce, 2001; Heil, 2004). However, to our knowledge, no study in climbing plant species has addressed the interaction between plant responses induced upon damage and the availability of light and support. Within the theoretical context of plant resource allocation (Zangerl and Bazzaz, 1992), the expression of both induced responses and plastic responses to abiotic factors should depend on the ecological scenario. On one hand, induced responses to herbivory are thought to reduce costs of plant defense (Strauss et al., 2002) and hence are expected to be greater in resource-poor environments (Gianoli and Niemeyer, 1996). On the other hand, induced responses divert plant resources and hence herbivory may limit phenotypic plasticity to challenging environments (Quezada and Gianoli, 2006).

The field bindweed, *Convolvulus arvensis*, as is the case in many taxa from the Convolvulaceae, is a twining vine. Plants of this species that grow erect, i.e., climbing onto physical support, exhibit morphological differences from those growing prostrate (Gianoli, 2001). For instance, climbing individuals have shorter internodes and petioles (Den Dubbelden and Oosterbeek, 1995; Gianoli, 2001), and shaded plants show longer internodes and petioles, and have thinner leaves (Gianoli, 2001; González and Gianoli, 2004). The stems and leaves of *C. arvensis* contain tropane alkaloids (TAs) (Todd et al., 1995), which are toxic against insects and mammals (Krug and Proksch, 1993; Todd et al., 1995) and are inducible after herbivory (Khan and Harborne, 1990). Interestingly, it was recently shown that simulated herbivory on this twining vine enhances its climbing success, and that climbing individuals in the field suffer less herbivory than prostrate ones (Gianoli and Molina-Montenegro, 2005).

The present greenhouse study in *C. arvensis* had two main goals. First, we evaluated the effect of the light environment and support availability on the induction of TAs after leaf damage. Thus, in view of the recently described damage-induced twining that may reduce herbivory (Gianoli and Molina-Montenegro, 2005), we considered another damage-induced response of potential relevance for plant defense, further inquiring into its environmental modulation. In particular, we evaluated the hypothesis that induced responses should be of greater magnitude in resource-poor environments. Second, we tested whether leaf damage modifies the phenotypic responses to shade and physical support that have been described for *C. arvensis* and another convolvulaceous twining vine (Gianoli, 2001, 2002, 2003). Thus, we aimed at determining whether herbivory may limit plant phenotypic plasticity. This is of interest for the debate on the effects of herbivory on plant fitness (Marquis, 1992). Thus, if foliar damage constrains the capacity of plants to display functional responses to the environment, then the consequences of herbivory may be magnified in a context of changing environments.

Methods and Materials

Convolvulus arvensis L. (Convolvulaceae), the field bindweed, is a perennial herb with slender stems (0.5–2 m long) that are trailing or twining when physical support is available. Leaves (2–6 cm long) are simple, alternate, triangular to ovate-oblong. This species is typically found in cultivated fields and roadsides (Weaver and Riley, 1982). In central Chile, *C. arvensis* is often attacked by a leaf beetle (Gianoli, unpublished data), and there are reports of intense herbivory by insects elsewhere (Rosenthal and Hostettler, 1980; Weaver and Riley, 1982).

Seeds of *C. arvensis* were collected on the edge of cereal fields in central Chile in April 1999 from more than 40 mother plants. After acid scarification, seeds were germinated in the dark at room temperature (24±2°C). Seedlings were initially planted in 300-ml plastic pots filled with potting soil and placed outdoors. Two wk after seedlings attained the second true leaf, they were transplanted into 5-l pots filled with potting soil, and half of them received physical support (see below). Eight wk later, plants were transferred to the greenhouse, and light treatments were started (see below). Experiments were performed during 5 mo (November 2002–March 2003) at maximum and minimum temperatures of 23±2°C and 10±2°C, respectively.

Eight experimental treatments (N=12 plants per treatment) were applied after a factorial array of 2 light treatments (sun and neutral shade), 2 support conditions (with and without physical support, i.e., climbing and prostrate), and 2 damage treatments (undamaged and simulated herbivory). Sun = plants growing in the greenhouse (average light intensity at noon: 1120 and 1176 μmol m^{-2} sec^{-1} PAR for prostrate and climbing plants, respectively); Shade = plants growing under a double-layered black cloth hung at 2 m over the greenhouse benches (average light intensity at noon: 55 and 98 μmol m^{-2} sec^{-1} PAR for prostrate and climbing plants, respectively). Neutral shade has been shown to elicit plant phenotypic responses similar to those caused by neighboring vegetation (Peñalosa, 1983; Lee, 1988; Gianoli, 2001). Light intensity was recorded with a Li-250 light meter (LiCor, Lincoln, NE, USA). Support consisted of a plastic stake (0.8 cm diam, 1.2 m long) vertically placed in contact with the stem of seedlings. Damaged plants suffered 25% defoliation with scissors, 50% of leaf area being removed in half of the leaves; leaves were clipped along the mid vein. Foliar damage was applied twice during the experiment: 7 and 11 wk after plants were transferred to the greenhouse. At each time that foliar damage was applied, we did it so that defoliation level was kept at 25%. The 48 plants of each of the two light treatments were placed alternately on the greenhouse benches and rotated every 2 wk. Plants were watered with tap water every second day.

One wk after the last foliar damage we measured in each plant the length of 3 internodes and 3 petioles at midshoot (40–50 cm over soil pot level), and the length, width, thickness, and elevation angle of 3 intact leaves at midshoot. The choice of experimental leaves was done regardless of the proximity to damaged leaves. Morphological measurements were

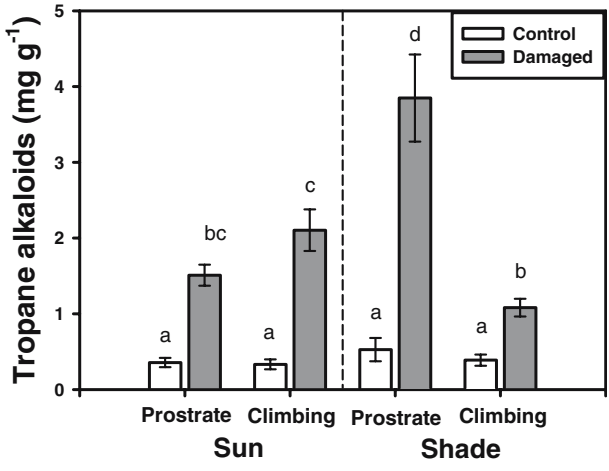


Fig. 1 Concentration of tropane alkaloids (mean ± SE) in leaves of the twining vine *Convolvulus arvensis* growing in different treatments of light intensity (sun and shade), support availability (prostrate and climbing) and damage (undamaged control and simulated leaf herbivory). Means sharing a letter are not significantly different (P>0.05, Tukey test)

Table 1 Three-way anova of tropane alkaloids and shoot traits in the twining vine *Convolvulus arvensis* (*F* ratios are shown)[a]

	Light	Support	Damage	L×S	L×D	S×D	L×S×D
Tropane alkaloids	2.84	**10.72**	**204.51**	**24.76**	0.25	**6.08**	**20.77**
Internodes length	**143.61**	**324.17**	**6.02**	**8.18**	**4.76**	0.16	7.52
Petiole length	**493.28**	**374.58**	**16.63**	**7.53**	**5.16**	0.33	0.36
Number of leaves	0.11	**28.59**	0.14	1.03	0.43	0.00	0.47
Leaf thickness	**194.43**	**49.03**	1.80	0.10	1.61	0.15	0.71
Leaf shape	**10.57**	**6.63**	2.83	**4.62**	**3.98**	0.27	0.01
Leaf angle	**1539.79**	**741.53**	0.61	**380.67**	2.61	0.08	0.44

Main factors were light (sun and shade), support (prostrate and climbing), and damage (control and mechanical leaf damage).

Leaf shape = length/width ratio; leaf angle = angle of the lamina with the horizon.

[a] *df*=1,70 for tropane alkaloids and 1,86 for the other traits. Significant effects are shown in boldface.

done with a digital caliper (Mitutoyo; resolution, 0.01 mm). To determine the concentration of TAs we randomly chose 10 plants per experimental group, and for each individual we harvested 15 g of leaf fresh biomass from the upper third of the plant. We included both damaged and intact leaves. Samples were shredded and macerated in methanol (85%) for 72 h. The methanolic extract was concentrated in a rotavapor (Heidolph Instruments OB2000, Schwabach, Germany) with a water bath at 40°C. Three consecutive extractions with dichloromethane were made after adding water, chloridric acid (to pH 1), and sodium carbonate (to pH 12), respectively. All three extractions were blended, and a 10-μl aliquot was directly injected into a high-performance liquid chromatograph (Shimadzu SCL-10A, Kyoto, Japan; UV detector Shimadzu SPD-M10A). Flow rate was 1 ml min^{-1}. The concentration of TAs, specifically of tropine ($C_8H_{15}NO$), which has been earlier detected in *C. arvensis* aerial biomass (Todd et al., 1995), was determined by using a commercial

Table 2 Morphological traits (mean±SE) of the twining vine *Convolvulus arvensis* in different treatments of light (sun and shade), support availability (prostrate and climbing), and damage (undamaged control and simulated leaf herbivory)[a]

	Sun				Shade			
	Prostrate		Climbing		Prostrate		Climbing	
	Control	Damaged	Control	Damaged	Control	Damaged	Control	Damaged
Internodes length (mm)	33.3±1.2 CD	35.2±1.4 BC	22.2±0.7 E	20.0±0.8 E	42.2±1.0 A	37.8±0.5 B	31.2±0.5 D	29.7±0.6 D
Petiole length (mm)	13.8±0.6 BC	13.1±0.6 C	7.8±0.5 D	7.2±0.2 D	23.3±0.6 A	21.4±0.8 A	15.8±0.3 B	13.1±0.3 C
Number of leaves	23.5±3.2 B	24.3±3.1 AB	32.3±1.9 AB	36.4±4.7 AB	21.7±2.7 B	22.7±2.7 B	38.8±5.0 A	36.4±2.7 AB
Leaf thickness ($mm \times 10^{-1}$)	3.75±0.1 BC	3.58±0.1 CD	4.36±0.1 A	4.12±0.1 AB	2.64±0.1 FG	2.53±0.1 G	3.07±0.1 EF	3.16±0.2 DE
Leaf shape	0.67±0.02 B	0.67±0.02 B	0.66±0.02 B	0.67±0.02 B	0.77±0.03 A	0.71±0.01 AB	0.70±0.02 AB	0.66±0.01 B
Leaf angle (°)	17.4±0.9 B	15.4±0.9 B	49.9±1.7 A	48.5±1.5 A	1.91±0.4 D	3.17±0.4 D	8.00±0.4 C	7.92±0.4 C

[a] Leaf shape = length/width ratio; leaf angle = angle of the lamina with the horizon. Means sharing a letter are not significantly different ($P>0.05$, Tukey test) within rows.

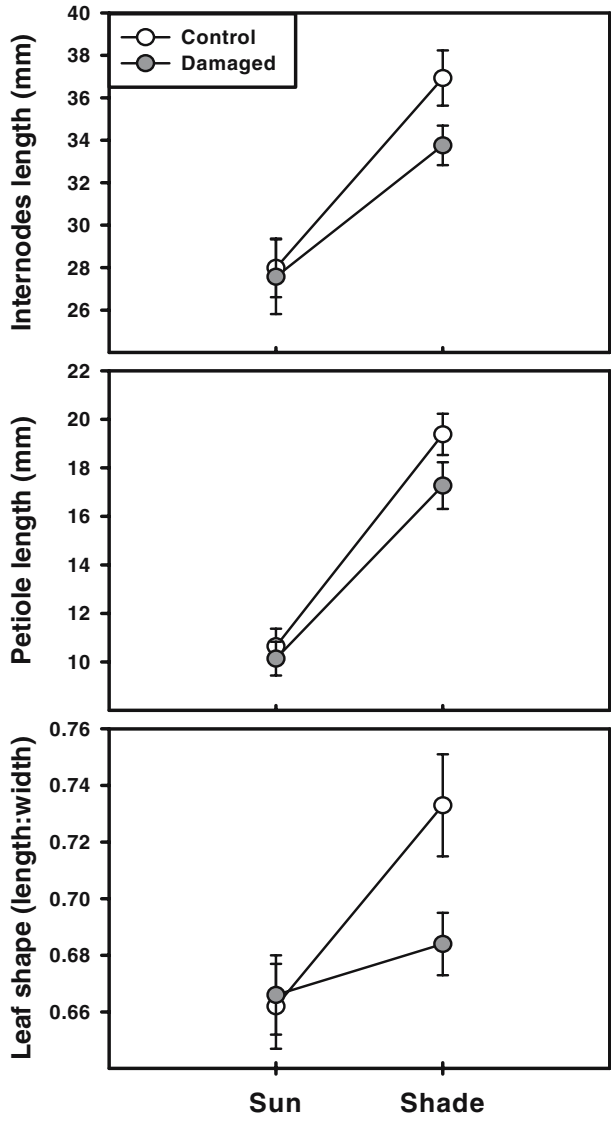


Fig. 2 Plasticity to shading in three morphological traits (mean ± SE) of undamaged (control) and clipped (damaged) plants of the twining vine *Convolvulus arvensis*. See Table 1 for the statistical significance of the Damage × Light interactions

tropine standard (Sigma-Aldrich Chemie, Steinheim, Germany). Detection of tropine was performed at 280 nm.

The effect of treatments on TAs and morphological traits was tested by a three-way ANOVA (main factors: Light, Support, and Damage). The significance of differences between means was determined by a Tukey test. To address the goals of the study, we examined the significance of interactions between Damage and the other main factors in the ANOVA. These results would show whether the light environment and support availability affect the induction of TAs after leaf damage, and whether leaf damage modifies the phenotypic responses of plants to shade and physical support.

Results

Overall, we did not find a significant effect of light or support treatments on the constitutive level of TAs, and there was a consistent pattern of induction of TAs after leaf damage in each environmental condition (Fig. 1). Although we failed to detect an effect of the light environment on the induction of TAs, there was a significant Support × Damage interaction, suggesting a differential effect of simulated herbivory on chemical defenses of prostrate and climbing plants (Table 1). However, it was clear that the latter was a result of the much greater induction of TAs observed in prostrate plants in the shade, whereas chemical induction was similar in prostrate and climbing plants growing in the sun (Fig. 1). Consequently, the main result for TAs with regard to the research goals was that there was a highly significant Light × Support × Damage interaction (Table 1). This means that the induction of TAs after leaf damage in *C. arvensis* was particularly affected by some combinations of light and support availability.

With the exception of leaf number, which was only affected by support, all the morphological traits evaluated responded to differences in the availability of both light and support (Table 1). Shorter internodes and petioles, and thicker leaves were found in climbing plants and in the sun (Table 2). Plants lacking support and growing in the shade showed a more ovate-oblong shape and a lower angle with the horizon (Table 2). Damage seemed to show a consistent effect on internodes and petiole length (Table 1). However, the tendency for damaged plants to have shorter internodes and petioles was statistically significant only in shaded and prostrate plants, and in shaded and climbing plants, respectively (Table 2). Interestingly, there were significant Light × Damage interactions for internodes and petiole length and leaf shape (Table 2). Particularly, damaged plants showed reduced plasticity to shading in these three traits in comparison with control, undamaged plants (Fig. 2).

Discussion

Induced responses to herbivory include short-term increases in chemical or physical defenses (Karban and Baldwin, 1997; Agrawal, 2005) and less rapid changes in resource uptake/allocation and growth patterns (Collantes et al., 1997; Strauss and Agrawal, 1999). In the case of climbing plants, there is evidence of changes in both chemical defenses and biomass allocation induced by herbivores (Schierenbeck et al., 1994; Fordyce, 2001). Recent findings in the twining vine *C. arvensis* (Gianoli and Molina-Montenegro, 2005) and related species (Atala and Gianoli, unpublished data) show that leaf damage induces an enhanced twining that, at least for *C. arvensis*, may reduce herbivory in the field (Gianoli and Molina-Montenegro, 2005). Induced defenses are thought to reduce costs of plant defense (Gianoli and Niemeyer, 1997; Heil, 2002; Heil and Baldwin, 2002; Strauss et al., 2002; Agrawal, 2005). Thus, greater induction might be expected in resource-poor or limiting environments compared with benign environments (Gianoli and Niemeyer, 1996). Accordingly, we found that vines showed the greatest induction of toxic TAs (Krug and Proksch, 1993; Todd et al., 1995) when damage occurred in a resource-poor environment (i.e., shade) and there was no cue (i.e., support) of future increase in resource uptake and/or escape from herbivores. This suggests that defensive responses in this vine species are adaptively tuned with the surrounding environment. It would be interesting to test how general is this induced responses pattern in climbing plants, particularly in those growing

in dynamic environments in terms of light and support availability such as rainforest understories.

Adaptive plant responses to shading and to herbivory have been studied widely and thoroughly, but there is little research on the interaction between plant phenotypic plasticity to both ecological factors (Cipollini, 2004). In *Chenopodium album*, induced responses to caterpillar herbivory did not affect subsequent responses to shading, and the magnitude of induced resistance (reduction of caterpillar mass) was similar in shaded and control light plants (Kurashige and Agrawal, 2005). Damaged plants of *Convolvulus demissus* are less plastic to experimental drought than undamaged control plants (Quezada and Gianoli, 2006). A study with genetically altered *Cucumis sativus* varieties reported some evidence that the constitutive expression of shade-avoidance response and resistance to herbivory are negatively related (McGuire and Agrawal, 2005). In the present study, damaged and hence induced vines showed reduced plasticity to shading in three morphological traits of functional significance for the enhancement of light capture. Plants receiving damage were less able to elongate internodes and petioles and to maximize leaf surface in comparison with undamaged plants. This shows a negative interaction between plant phenotypic responses to biotic and abiotic factors, as theory predicted (Cipollini, 2004). It is, therefore, verified that herbivory, in addition to its capacity to reduce plant fitness, may directly limit adaptive plant responses to the environment. Consequently, in the context of plant competition, (induced responses to) herbivory may affect the outcome of plant–plant interactions.

The evolution of a climbing habit constitutes a key innovation in flowering plants (Gianoli, 2004). Climbing plants are a typical element of rainforests. Despite the vast body of knowledge on herbivory in rainforests, the interaction between herbivory and the functional ecology of climbing plants has received little attention. This overlooked research line might provide insights into the current patterns of distribution and abundance of vines (Wright et al., 2004) as well as explanatory hypothesis for their evolutionary success (Gianoli, 2004).

Acknowledgement We thank Z. Rozas, S. Meneses, and V. Hernández for assistance during the chemical analysis, and M. González and P. Torres for help in the greenhouse. M. Heil and an anonymous reviewer made helpful suggestions for improvement of the manuscript.

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J Chem Ecol (2007) 33:105–114
DOI 10.1007/s10886-006-9202-0

Interaction Between Atmospheric CO_2 and Glucosinolates in Broccoli

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Received: 22 June 2006 / Revised: 16 August 2006 / Accepted: 5 September 2006 /
Published online: 13 December 2006
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Abstract Total and individual glucosinolate contents of broccoli cv Marathon were assessed at ambient CO_2 (430–480 ppm) and elevated atmospheric CO_2 (685–820 ppm) to determine the ecological relationship between changing atmospheric CO_2 concentrations and phytochemicals. Elevated atmospheric CO_2 concentration had a differing effect on individual glucosinolates and glucosinolate groups. Total glucosinolate content increased at elevated atmospheric CO_2 concentration as a result of a strong increase in both methylsulfinylalkyl glucosinolates glucoraphanin and glucoiberin. In contrast, indole glucosinolates simultaneously decreased, predominantly because of a reduction of glucobrassicin and 4-methoxy-glucobrassicin contents. We conclude that changes in N content and N/S ratios as well as alterations in photochemical processes at elevated atmospheric CO_2 concentration can influence total and individual glucosinolates contents of *Brassicaceae*, as demonstrated in the greenhouse, for broccoli.

Keywords Carbon dioxide · *Brassica oleracea* var. *italica* · Alkyl glucosinolate · Indole glucosinolate · Chlorophyll fluorescence

Introduction

Glucosinolates are found exclusively in plants of the order Capparales, including horticultural important crop plants of the *Brassicaceae* family (Halkier and Du, 1997). They are bioactive compounds responsible for many physiological effects functioning as feeding deterrents and protective compounds against herbivores (Mewis et al., 2005). In addition, they are important as flavor compounds and cancer-preventive agents (Mikkelsen et al., 2002).

Glucosinolates are known to confer health-promoting effects because of their assumed anticarcinogenic properties (Holst and Williamson, 2004). Broccoli is a *Brassica* vegetable

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that has been recognized to be one component of a healthy diet, predominantly because of its high phytochemical content, in particular, glucosinolates (Brown et al., 2002; Schonhof et al., 2004; Zhang, 2004). With regard to current cancer incidence and global climate changes, it is of interest to determine to which extent glucosinolates might be affected by changing environmental factors, as glucosinolate content is influenced by numerous preharvest factors (Dekker and Verkerk, 2003). *Brassica* vegetables grown during different seasons exhibited varying glucosinolate contents, thereby establishing a distinct influence of environmental factors, such as temperature and radiation, on them (Shattuck and Wang, 1994; Schonhof et al., 1999; He et al., 2000; Krumbein and Schonhof, 2001; Schreiner, 2005). For example, the contents of glucoraphanin and glucoiberin in broccoli inflorescences increased at daily mean temperatures between 7 and 13°C combined with a moderate daily mean radiation of 10 to 13 mol m^{-2} d^{-1}, whereas the main indole glucosinolate glucobrassicin was reduced (Schonhof et al., in press). Atmospheric carbon dioxide concentration represents an environmental factor that is currently undergoing great changes from 280 ppm in preindustrial times to approximately 365 ppm at present (Craigon et al., 2002; Krupa, 2003), with peaks in urban areas of up to 480 ppm (Pataki et al., 2006). Climate simulations indicate a further ongoing increase up to double of today's concentration (Gerber et al., 2004; Norby and Luo, 2004). Increases in CO_2 concentrations have implications for agriculture and the environment as a whole. For example, elevated CO_2 levels enhance photosynthesis, leading to vigorous plant growth and higher yield (Maevskaya et al., 1990; Wurr et al., 1998, 2000; Amthor, 2001), which is a phenomenon also observed in *Brassica* vegetables (Mishra et al. 1999).

Glucosinolates are nitrogen- and carbon-containing compounds, and thus, could be affected by atmospheric CO_2 enrichment, as this enhances the plant's carbon supply, thereby diluting its nitrogen content (Cotrufo et al., 1998). In addition, synthesis of amino acids—the precursors of the amino acid-derived glucosinolates—from triosephosphates has been observed to increase because of increasing photosynthesis at elevated CO_2 (Habash et al., 1995), hence, affecting glucosinolate content. However, at present, only little information is available on the effect of elevated atmospheric CO_2 on glucosinolate content in *Brassica* vegetables. Karowe et al. (1997) and, more recently, Reddy et al. (2004) studied the CO_2 concentration-induced effect on glucosinolate content in *Brassica* seedling leaves; however, mature leaves, roots, and inflorescences, which are key components of the human diet, were not investigated.

Because of the problems resulting from dramatic and continuing increase in atmospheric CO_2 concentrations and unknown effects on plant physiology, including glucosinolate responses to elevated atmospheric CO_2 concentration, the objective of the present study was to determine the effect of ambient and elevated atmospheric CO_2 on total and individual glucosinolate contents in mature broccoli inflorescences. For this purpose, broccoli was chosen as a model crop because of its high level of aliphatic and indole glucosinolates compared to other *Brassicaceae* (Schreiner, 2005).

Methods and Materials

Plant Material and Experimental Design

Three experiments were carried out in a block design, each with three replicates: experiment I with planting on 10 September 2003 and harvest at the end of November

2003; experiment II with planting on 9 January 2004 and harvest at the end of March 2004; and experiment III with planting on 5 February 2004 and harvest at the end of April 2004. All experiments were conducted in a controlled greenhouse environment. Daily mean temperature and daily mean radiation were calculated from measurements taken every 2 and 5 min, respectively.

For each treatment and replication, 54 broccoli plants (*Brassica oleracea* var. *italica* Plenck) cv Marathon were used, with each plant grown in a soil-filled 40-l container spaced 0.6×0.7 m, resulting in a density of 2 plants m^{-2}. Plants at the three-leaf-stage were exposed to two different atmospheric CO_2 concentrations: ambient atmospheric CO_2 (430–480 ppm) or elevated atmospheric CO_2 (685–820 ppm).

Plants were grown between 14 and 15°C with radiation between 6.23 and 10.04 mol m^{-2} d^{-1}. Artificial radiation was applied with high pressure sodium lamps (SONT-T 400W, Philips) to compensate for the lower solar radiation in winter time. Plants were illuminated from sunrise to 20:00 hours when solar radiation in the greenhouse was <200 μmol m^{-2} s^{-1}.

After soil analysis, because of the nutrient demand of the broccoli, plants were fertilized twice: first at the 10-leaf stage and then after head induction. Plants were irrigated with tap water distributed via drip tubes after soil moisture was measured (soil moisture probes: FP/m; measuring device: FOM/m; Easy Test Ltd, Lublin Poland). Biological pest management was performed when necessary with common green lacewing (*Chrysoperla carnea*).

Chlorophyll Fluorescence

To determine changes in plant photochemistry, which could lead to changes in glucosinolate content, chlorophyll fluorescence parameters were measured. This was measured for six plants for each atmospheric CO_2 concentration. After 20 min of dark adaptation, chlorophyll fluorescence was measured for the two fullest developed leaves (four measurements per leaf from the upper part of the leaf area) nearest to the broccoli head and for the broccoli inflorescences (six measurements per head) using a Mini-Pam (Walz, Effeltrich, Germany). To assess the minimal fluorescence (Fo), a pulse-modulated actinic light source of about 0.02 μmol m^{-2} s^{-1} was used (Van Kooten and Snel, 1990). For the maximal fluorescence (Fm), a saturating light pulse (duration 30 s, intensity 3,000 μmol m^{-2} s^{-1}) was applied. This procedure was repeated after light adaptation, and the current fluorescence (F) and maximal fluorescence (Fm′) were then measured. The following derived functions were used to calculate the chlorophyll fluorescence values (Genty et al., 1989):

$$\text{Photochemical quenching coefficient qP} = (\text{Fm}' - F)/(\text{Fm}' - \text{Fo}) \quad (1)$$

$$\text{Nonphotochemical quenching coefficient qN} = (\text{Fm} - \text{Fm}')/(\text{Fm} - \text{Fo}) \quad (2)$$

Sample Preparation

To determine glucosinolate content, fully developed broccoli heads were harvested. Fresh matter taken from the heads was directly measured after harvest. A mixed sample of florets of each replicate was taken consisting of five heads. The plant material was immediately deep-frozen (−28°C), then freeze-dried before being finely ground. This dry matter (dm) was used for glucosinolate, N, and S analyses.

Compound Analyses

The high-performance liquid chromatography (HPLC) method reported by Krumbein et al. (2005) was used for glucosinolate determination. Duplicates of freeze-dried sample material (0.5 g) were heated to and incubated at 75°C for 1 min, extracted with 4 ml of a methanol/water mixture (v/v=7:3, T=70°C), and after adding 1 ml of 0.4 M barium acetate, centrifuged at 4,000 rpm for 10 min. For an internal standard, 200 µl of a 5-mM stock solution of sinigrin in methanol were added to one of the duplicates just before the first extraction. The residue was extracted twice with 3 ml of the methanol/water mixture (v/v=7:3, T=70°C). Supernatants were pooled and made up to 10 ml with the methanol/water mixture (v/v=7:3, T=70°C). From this, 5 ml of the extract were applied to a 250-µl DEAE-Sephadex A-25 ion-exchanger (acetic acid-activated, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and rinsed with 10 ml bidistilled water. Next, 250 µl of a purified solution of aryl sulfatase (Boehringer-Mannheim GmbH, Mannheim, Germany) were applied and left for 12 hr before the desulfocompounds were flushed with 5 ml of bidistilled water.

Desulfoglucosinolate analysis was conducted by HPLC (Merck Hitachi) using a Spherisorb ODS2 column (5 µm, 250×4 mm). A gradient of 0–20% acetonitrile in water was selected from 2 to 34 min, followed by 20% acetonitrile in water until 40 min, and then 100% acetonitrile for 10 min until 50 min. Determination was conducted at a flow of 1.3 ml min^{-1} and a wavelength of 229 nm. Desulfoglucosinolates were identified by HPLC–atmospheric pressure chemical ionization–mass spectrometry[3] using the Agilent 1100 series in the positive ionization mode (Krumbein et al., 2005). Glucosinolate content was calculated using sinigrin as internal standard and the response factor of each compound relative to sinigrin. Determination of glucosinolates was performed in duplicate.

For the nitrogen (N) and sulfur (S) analyses, finely ground samples were burned in the element analyzer Vario EL using Dumas' burning method (VDLUFA, 1991). Assessment of total N and S contents was also performed in duplicate.

Statistical Analyses

Results were evaluated using analysis of variance (ANOVA) and regression analysis. Using ANOVA, differences between treatments were analyzed by Tukey's HSD test (HSD, Honest Significant Difference). Linear regression analyses were performed with one independent variable x using the linear model $y = a + bx$. All tests were performed at a significance level of $P<0.05$. Calculations were carried out using the software package Statistica™ for Windows™ (version 6.1, Statsoft Inc., Tulsa, OK, USA).

Results and Discussion

The total glucosinolate content and the contents of individual methylsulfinylalkyl glucosinolates glucoraphanin and glucoiberin and the indole glucosinolates glucobrassicin, neoglucobrassicin, 4-methoxy-glucobrassicin, and 4-hydroxy-glucobrassicin were quantitatively determined in broccoli inflorescences. The predominant glucosinolate was glucoraphanin (Table 1).

Elevated atmospheric CO_2 concentration affected the contents of both individual glucosinolates and glucosinolate groups. Total glucosinolate content increased at elevated atmospheric CO_2 concentration because of the rise in methylsulfinylalkyl glucosinolates,

Table 1 Total and individual glucosinolate content (μg g^{-1} dm) in broccoli inflorescences cv Marathon grown at ambient CO_2 (430–480 ppm) and elevated CO_2 (685–820 ppm)

	Ambient CO_2	Elevated CO_2	HSD	*P* Value	*F* Value
Methylsulfinylalkyl glucosinolates	3.75	5.13	0.58	0.002[a]	14.73
Glucoiberin	0.52	0.71	0.07	0.007[a]	9.86
Glucoraphanin	3.23	4.42	0.53	0.002[a]	14.92
Indole glucosinolates	2.70	2.24	0.35	0.161	2.17
Glucobrassicin	1.82	1.45	0.23	0.206	1.75
Neoglucobrassicin	0.69	0.62	0.15	0.495	0.49
4-hydroxy-glucobrassicin	0.02	0.02	0.01	0.686	0.17
4-methoxy-glucobrassicin	0.17	0.14	0.02	0.241	1.49
Total glucosinolates	6.46	7.37	0.77	0.013	8.05

Sample size for each CO_2 treatment $N=9$; degrees of freedom $N=15$; critical F value 3.68

[a] Statistically significant

glucoraphanin, and glucoiberin. In contrast, indole glucosinolates simultaneously decreased, predominantly because of a sharp reduction in glucobrassicin and 4-methoxy-glucobrassicin (Table 1). Because the increase in the methylsulfinylalkyl glucosinolate content was stronger than the decrease in indole glucosinolates, overall total glucosinolate content was higher in broccoli inflorescences at elevated atmospheric CO_2 compared to ambient CO_2 (Table 1).

Apart from the effect of CO_2 concentration on glucosinolate content and composition, however, plant species is thought to play a role. As shown by cabbage, which also belongs to *B. oleracea*, there was a decrease in indole glucosinolate content. Aliphatic glucosinolate content remained unaffected at elevated concentrations (720 ppm) (Reddy et al., 2004). In contrast, other *Brassica* genotypes showed different CO_2 responses in glucosinolate content changes. In leaves of oilseed rape (*Brassica rapa* subsp. *oleifera*), there was a general increase in indole glucosinolate and aromatic gluconasturtiin contents. The aliphatic glucosinolate content consisting mainly of alkenyls and hydroxyalkenyls remained unchanged under enriched CO_2 conditions (720 ppm) (Reddy et al., 2004). Further, total glucosinolates in leaves of radish (*Raphanus sativus*) (major alkenyl glucosinolate: glucoraphasatin) and turnip (*Brassica rapa* subsp. *rapa*) (major alkenyl glucosinolates: gluconapin and glucobrassicanapin), appeared to be unaffected with rising atmospheric CO_2 level (724 ppm) (Karowe et al., 1997).

Moreover, the changing N content and N/S ratio in broccoli inflorescences under different atmospheric CO_2 concentrations could influence contents of total and individual glucosinolates. As demonstrated in both C3 and C4 plants, N is reduced in plant tissue under elevated CO_2 regimes (Cotrufo et al., 1998). We found that in broccoli inflorescences, N content was reduced by 10.1%, leading to a decrease in the N/S ratio at elevated CO_2 levels (Table 2); this was correlated to pronounced changes in total and individual glucosinolate contents (Table 3). N content decrease was not because of a dilution effect because fresh and dm of broccoli heads did not significantly differ between CO_2 regimes (Table 2). In contrast, the S content in broccoli inflorescences was unaffected by rising CO_2 levels (Table 2). However, in oak leaves—exposed to 700 ppm CO_2—diminished S contents as a result of decreased adenosine 5′ phosphosulfate reductase activity have been reported (Kopriva and Rennenberg, 2004), indicating genotypic differences in the acclimation of sulfate assimilation at elevated atmospheric CO_2 concentration.

Table 2 Inflorescence parameters of broccoli cv Marathon grown at ambient CO_2 (430–480 ppm) and elevated CO_2 (685–820 ppm)

Parameter	Ambient CO_2	Elevated CO_2	HSD	*P* Value	*F* Value
S content (% dm)	0.78	0.77	0.04	0.513	0.45
N content (% dm)	3.87	3.48	0.14	0.022[a]	6.52
N/S ratio	4.98:1	4.54:1	0.25	0.042[a]	4.94
Dry matter (%)	11.55	11.83	0.38	0.593	0.30
Fresh matter per head (g)	298.69	319.03	31.53	0.494	0.40

Sample size for each CO_2 treatment N=9; degrees of freedom N=15; critical F value 3.68

[a] Statistically significant

For aliphatic glucosinolate biosynthesis, sulfate assimilation in the form of cysteine, which is a key source of methionine-derived aliphatic glucosinolate, is a crucial step. Thus, the contents of the methylsulfinylalkyl glucosinolates glucoraphanin and glucoiberin increased with decreasing N content and decreasing N/S ratio at elevated CO_2 levels (Fig. 1, Table 3). The more the N/S ratio decreases because of diminished N content, the stronger the synthesis of sulfurous cysteine as a precursor of methionine seems to be (Hesse et al., 2004). Moreover, cysteine and methionine act as effective sulfur donors in thiohydroxamate formation in the synthesis of aliphatic and indole glucosinolates (Wetter and Chisholm, 1968; Mikkelsen et al., 2002); however, in our study, the indole glucosinolates glucobrassicin and 4-methoxyglucobrassicin decreased with reduced N content and reduced N/S ratio at elevated CO_2 levels (Fig. 1, Table 3). We assume that the synthesis of tryptophan-derived indole glucosinolates is not so strongly sulfur-dependent as the synthesis of methionine-derived alkyl glucosinolates, thereby explaining the positive correlation between indole glucosinolate content and N/S ratio and N content, respectively.

Changes in broccoli photochemistry could be another reason for the CO_2 concentration-induced effect on glucosinolates. Enhanced photochemical activity can result in the enhanced formation of ATP and reduction equivalents necessary for fixation and assimilation of CO_2 in the Calvin cycle, leading to the formation of a carbohydrate pool that forms the basis for the synthesis of further compounds, such as amino acids (Larcher,

Table 3 Correlation between total glucosinolate content, individual glucosinolate content, S content, N content, and N/S ratio in broccoli inflorescences cv Marathon

Correlation	Beta	R^2	*P* Value	*F* Value
N/S ratio per methylsulfinylalkyl glucosinolates	−0.56	0.31	0.019[a]	6.85
N content per glucoiberin	−0.49	0.24	0.048[a]	4.65
N/S ratio per glucoiberin	−0.37	0.13	0.148	2.32
N content per glucoraphanin	−0.56	0.32	0.019[a]	6.92
N/S ratio per glucoraphanin	−0.58	0.34	0.014[a]	7.73
N/S ratio per indole glucosinolate	0.70	0.49	0.002[a]	14.55
N content per glucobrassicin	0.88	0.78	<0.001[a]	52.42
N/S ratio per glucobrassicin	0.82	0.68	<0.001[a]	31.60
N content per 4-methoxy-glucobrassicin	0.81	0.65	<0.001[a]	27.70
N/S ratio per 4-methoxy-glucobrassicin	0.68	0.46	0.003[a]	12.59
N/S ratio per total glucosinolates	0.01	0.00	0.959	0.003

Sample size N=18; degrees of freedom N=15; critical F value 3.68

[a] Statistically significant

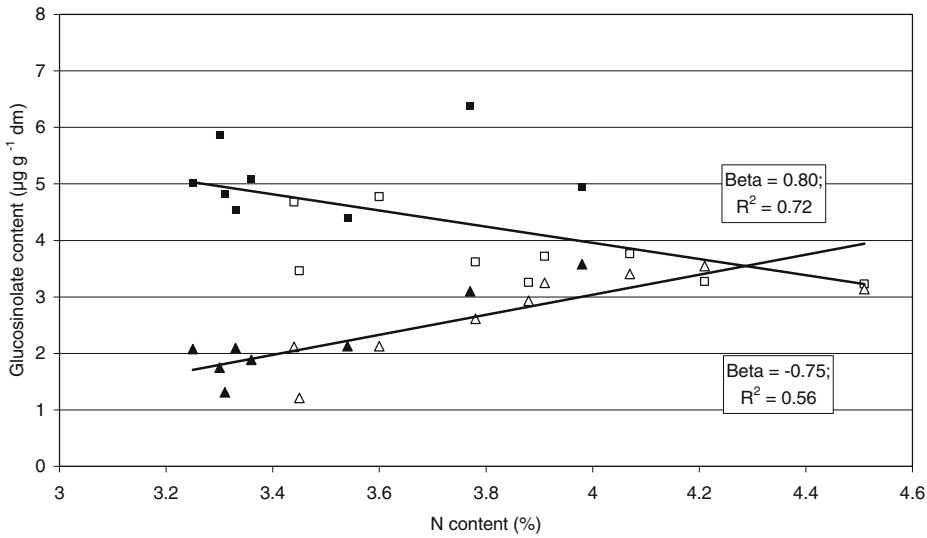


Fig. 1 Correlation between N content and methylsulfinylalkyl and indole glucosinolates in broccoli inflorescences cv Marathon. CO_2 □ Methylsulfinylalkyl glucosinolates at ambient CO_2. ■ Methylsulfinylalkyl glucosinolates at elevated CO_2. Δ Indole glucosinolates at ambient CO_2. ▲ Indole glucosinolates at elevated CO_2. Values are the means of three measurements in duplicate

1994). Thus, enhanced amino acid formation could promote glucosinolate synthesis because glucosinolates are amino acid-derived compounds (Grubb and Abel, 2006).

With elevated CO_2 concentration in both plant organs—inflorescences and leaves—a greater portion of the absorbed light apparently was used for photochemical purposes, as indicated by the enhanced photochemical quenching coefficient (qP) at harvest date (Fig. 2), as well as that observed throughout the entire development of the broccoli head (data not shown). Simultaneously, as shown by the nonphotochemical quenching coefficient (qN), the portion of the absorbed photons diverting into the nonphytochemical pathway remained unaffected by varying CO_2 concentrations (Fig. 2). With a rising qP of broccoli leaves, indole glucosinolate content increased in the inflorescence tissue. In addition, rising qP values up to an optimum range of 0.4–0.5 were accompanied with increasing methylsulfinylalkyl glucosinolate content (Fig. 3), whereas no correlation was found between indole and methylsulfinylalkyl glucosinolate contents and the qP of the broccoli inflorescences (data not shown). This result demonstrates the large source capacity of the leaves in comparison with the inflorescences because of the pronounced photoassimilating leaf area.

Because glucosinolates are also formed in leaves of broccoli (Scheuner et al., 2005) and, as shown in *Arabidopsis thaliana*, are transported via the phloem (Chen et al., 2001; Chen and Andreasson, 2001), a glucosinolate exchange between individual plant organs is possible. In *A. thaliana*, the pattern of glucosinolate distribution corresponded to the transport of photoassimilates, which in turn was consistent with glucosinolate translocation in the phloem following the source–sink relationship (Chen et al., 2001). We assume that in broccoli, glucosinolate transport from leaves to inflorescences was affected by a changing source–sink relationship induced by enhanced demand for soluble carbohydrates of the stronger growing broccoli head (at the earlier stage of head formation, data not shown) at

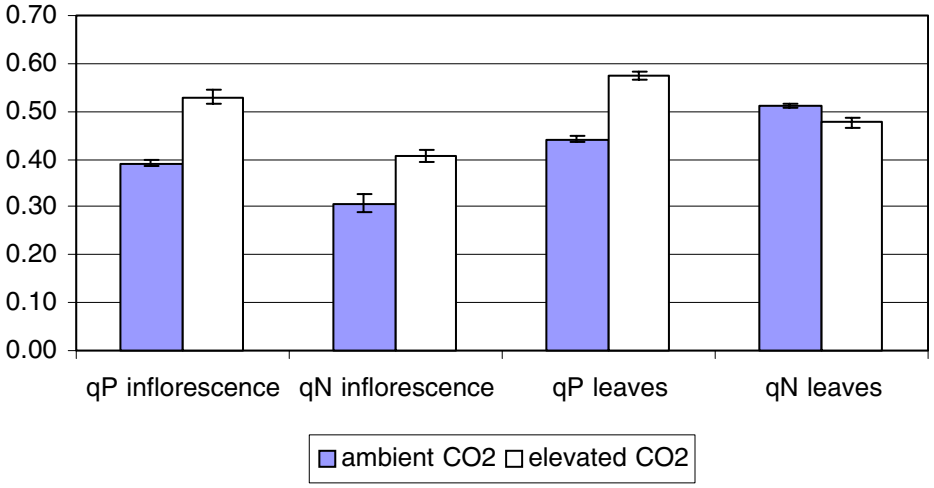


Fig. 2 Photochemical quenching coefficient (qP) and nonphotochemical quenching coefficient (qN) of broccoli inflorescences and leaves cv Marathon grown at ambient CO_2 (430–480 ppm) and elevated CO_2 (685–820 ppm). Values are the means of 36 (inflorescences) and 48 measurements (leaves), respectively. The bars represent the standard error

elevated CO_2. This would account for the especially enhanced methylsulfinylalkyl glucosinolate content in the inflorescences.

In summary, our results provide evidence that, in respect to predicted elevation of atmospheric CO_2 concentrations, secondary plant metabolism is affected by increasing CO_2 levels as demonstrated by glucosinolates. We have further demonstrated that different glucosinolate groups (methylsulfinylalkyl and indole) and individual glucosinolate contents

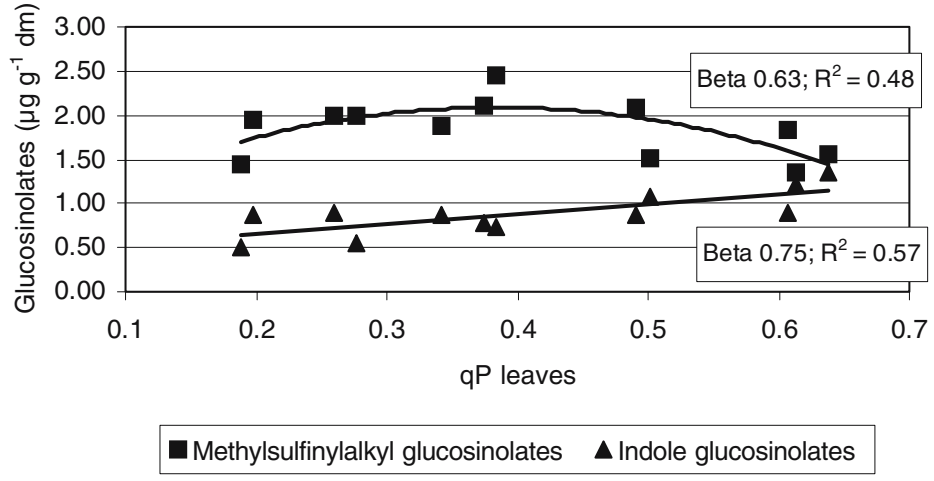


Fig. 3 Glucosinolate content and photochemical quenching coefficient (qP) of broccoli leaves cv Marathon grown at ambient CO_2 (430–480 ppm) and elevated CO_2 (685–820 ppm). Values are the means of 48 measurements. For the methylsulfinylalkyl glucosinolates as linear model $y = a + bx + cx^2$ was used; for the indole glucosinolates as linear model $y = a + bx$ was used

need to be assessed to have a clear picture of a plant's response to elevated CO_2 concentrations. We think that the CO_2 concentration-induced effect may be different for each *Brassica* species, and that changes in N content and N/S ratios as well as alterations in photochemical processes because of increased C content could influence the plant's total and individual glucosinolate contents.

Atmospheric CO_2 is both an important environmental and anthropogenic factor. It can enhance the health-promoting quality of broccoli because of induced glucosinolate content changes. Moreover, glucosinolates are involved in plant defense response against pathogen and herbivore attack (Mikkelsen et al., 2003). Because the variation in chemical defense by synthesis of structurally different aliphatic, aromatic, and indole glucosinolates can vary with the pathogen/herbivore (Mewis et al., 2005), changing of individual glucosinolate content by increasing atmospheric CO_2 levels could also result in a changing susceptibility to pathogen and herbivore attacks.

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J Chem Ecol (2007) 33:115–129
DOI 10.1007/s10886-006-9206-9

The Auxin-like Activity of Humic Substances is Related to Membrane Interactions in Carrot Cell Cultures

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Received: 2 February 2006 / Revised: 24 May 2006 / Accepted: 14 September 2006 /
Published online: 17 November 2006
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Abstract A detailed characterization of two humic fractions was performed: One with low relative molecular mass (LMr<3,500 Da) and one with high relative molecular mass (HMr >3,500 Da). Distinct ^{1}H NMR spectroscopic patterns were observed for the two fractions. HMr showed an aromatic proton region, an intense and broad region (3.0–5.0 ppm) attributed to sugar-like and polyether components, and an intense doublet at 1.33 ppm (identified as protons of the β-CH_3 in lactate). In contrast, LMr did not show resonances due to aromatic protons and was characterized by a broad unresolved region, assigned to sugar-like components. The ^{13}C NMR spectra showed that the LMr humic fraction was richer in carboxylic and aliphatic C groups compared to HMr fraction. These substances were fluorescein-labeled [fluorescein isothiocyanate (FITC)], and their interaction with carrot cells in culture was monitored for 10 d, and compared to FITC–indole-3-acetic acid (IAA) to clarify their mechanisms of biological activity. After different incubation times, fluorescein staining of carrot cells and decrease of fluorescein concentration in the culture medium were evaluated. Fluorescent membrane staining was only present in IAA and the LMr humic fraction treated cell cultures. A consequential decrease of fluorescein concentration in the culture media was also observed. Pretreatment of carrot cells with unconjugated IAA or LMr humic fraction markedly reduced fluorescein staining of both FITC–IAA and FITC–LMr humic fraction. Blocking tests gave indirect evidence of

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possible binding of the LMr humic fraction to IAA cell membrane receptors. These results indicate that the two humic fractions behave differently. Only LMr humic fraction, like IAA, interacts with cellular membranes in carrot cell cultures.

Keywords Auxin-like activity · Biological activity · Carrot cell cultures · DRIFT · ELISA · Fluorescence · GC-MS · Humic substances · ^{13}C-NMR · ^{1}H-NMR

Introduction

Humic substances (HS), the major components of soil organic matter, have multiple roles in plant growth (Tan, 1998), and are the subject of study in various areas of agriculture, such as soil chemistry, fertility, plant physiology, and environmental sciences. Many papers have shown that HS may improve physical and chemical soil properties, favor a higher concentration of ions in soil solution, and act as source and sink for nutrients, such as P, N, and K (Vaughan et al., 1985). In the rhizosphere, interaction between the root system and humic matter is possible when humic molecules present in the soil solution are small enough to flow into the apoplast and reach the plasma membrane. This event occurs in the vicinity of the root surface, where release of organic acids by roots enable dissociation of the humic macrostructure, and subsequent release of the otherwise unavailable bioactive fractions (Dell'Agnola and Nardi, 1987; Piccolo et al., 1992).

Many studies have shown that soil humic matter can affect plant growth and metabolism, by regulating plant biochemical and physiological processes (Vaughan et al., 1985), by nutrient absorption (Clapp et al., 2001; Varanini and Pinton, 2001), by enzyme activation or inhibition (Muscolo et al., 1993, 1996), and by inducing morpho-functional changes in root architecture (Canellas et al., 2002). In many cases, these effects are comparable to that induced by some plant growth regulators, e.g., the auxins. The complex biological activity of humic matter depends on its concentration, chemical characteristics, and molecular size and weight (Maggioni et al., 1987; Albuzio et al., 1993; Nardi et al., 1994; Muscolo and Nardi, 1997; Muscolo et al., 1998).

Muscolo et al. (1993, 1996) have shown that humic substances induce morphogenetic and biological changes in leaf explants of *Nicotiana plumbaginifolia*. They affect the patterns of peroxidase and esterase, enzymes that are involved in organogenesis, and may be indicators of somatic embryogenesis. These effects, peculiar to humic fraction with a low relative molecular mass (<3,500 Da), were similar to those produced by indole-3-acetic acid (IAA); whereas the humic fraction with a high relative molecular mass (>3,500 Da) had no effect on this system. A study on homogeneous carrot (*Daucus carota*) cell cultures compared the effects of the low relative molecular mass humic fraction to different auxins (Muscolo et al., 1999). This humic fraction caused an increase in carrot cell growth similar to that induced by 2,4-dichlorophenoxyacetic acid (2,4-D) and promoted morphological changes similar to those induced by IAA. The different biological activities of humic fractions, with high and low relative molecular mass, may be due to their chemical heterogeneity, polydisperse nature, and/or different conformational arrangements.

The mechanism by which soil HS stimulate plant biological activity is not yet clarified; this is, in part, due to the heterogeneity and complexity of HS that poses a serious impediment for characterization. Thus, attempts to relate humus structure to biological activity have produced contrasting results. The chemical structure of HS is not well-known. However, the main structure of humic matter has a polycyclic aromatic core linked with

side chain structures such as carbohydrates, phenolics, proteins, peptides, and metal ions (Cheshire et al., 1967). Considering that the genesis of HS involves combinations of several reaction pathways and a wide variety of chemical binding systems, it is difficult to define a clear concept of their composition (Hayes, 1997). Many of the original classical methods to understand the nature of HS were based on elemental composition, but the results obtained represent averages for agglomerations of molecules, and it is impossible to derive precise empirical formulae from these data. It is impossible to break the HS by hydrolysis or other chemical degradation techniques without producing denaturated substances, causing artificial results.

Considerable progress has been made in the last few years regarding some of the gross features of HS, by employing various spectroscopic procedures: infrared spectroscopy, electron spin resonance, Raman, ultraviolet-visible, fluorescence, and X-ray photoelectron spectroscopy. Considerable effort has been focused on ^{13}C-NMR and ^{1}H-NMR spectroscopy, the most powerful tools available to determine the structure of both organic and inorganic species.

In this study, two humic fractions with different relative molecular mass were extensively characterized by means of different spectroscopic techniques [diffuse reflectance infrared Fourier transforms (DRIFT), ^{13}C, ^{1}H nuclear magnetic resonance (NMR), and gas chromatography-mass spectroscopy (GC-MS)], for a better understanding of their chemical structure and physical–chemical properties.

These humic fractions were derivatized with fluorescein to observe their interaction with carrot cells in culture, with the aim of clarifying the mechanism of their biological activity.

Methods and Materials

Study Sites The study was carried out in the Ah horizon of a Calcaric Cambisol (CMc-F.A.O. classification) (FAO-UNESCO, 1990) of uncultivated couch grass (*Agropyron repens* L.) fields, located in the College of Agriculture Farm (University of Padua).

Preparation of Humic Fractions Humic substances were extracted from the air-dried samples with 0.1 N KOH (1:20 w/v) at room temperature for 16 hr under a N_2 atmosphere. Suspended material was removed by centrifugation at 7,000×*g* for 20 min. Humic extracts were transferred into 18,000 mol. wt cut-off dialysis tubing Visking (Medicell, UK) and dialyzed against double-distilled water. The retained solution was desalted by ion exchange on Amberlite IR 120 (H^+ form) and the pH adjusted to pH 2.1 with glacial acetic acid. The acidified solution was dialyzed through 3,500 mol. wt. cut-off Spectra/Por® 3 tubing (Spectrum, Gardena, CA) against distilled water. This procedure separated the total humic extract into high (>3,500 Da) and low (<3,500 Da) relative molecular mass (Mr) fractions. The HMr and LMr fractions were collected inside and outside the dialysis tubing, respectively. These solutions were freed from unreacted acetic acid and reduced in volume to about 50 ml by vacuum distillation (Nardi et al., 1991).

Diffuse Reflectance Infrared Fourier Transform (DRIFT) Spectroscopy For each analysis, 2 mg of dried sample was mixed with 148 mg KBr (FT-IR grade, Aldrich Chemical, Milwaukee, WI), so that the mixture became homogeneous. After grinding, the sample mixture was heaped over the top of the microsample cup. Any excess material was removed with a straight-edged tool. For the background, a microsample cup of pure KBr was

prepared. DRIFT spectra were recorded with 200 scans collected at 4 cm^{-1} resolution using a Nicolet Impact 400 FT-IR Spectrophotometer (Madison, WI) and fitted with an apparatus for diffuse reflectance (Spectra-Tech., Stamford, CT). DRIFT spectra are the average of 200 scans and the resolution was set at 4 cm^{-1} resolution.

^{1}H NMR Spectroscopy For each fraction, 20 mg of sample was dissolved in 0.5 ml D_2O (deuterium oxide). Spectra were recorded with a Bruker ACF 250 spectrometer (Karlsruhe, Germany) using a 5-mm multinuclear probe. ^{1}H spectra were accumulated with a 16-K data point, a one-pulse sequence, a 40°-pulse angle, a 3-sec relaxation delay, and a sweep width of 2.5 kHz. To obtain a satisfactory signal-to-noise ratio, 1,000–2,000 scans were needed. Gated irradiation was applied between acquisitions to presaturate the residual water peak. 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt was added to the samples to provide a chemical shift standard. Spectra were divided into three main regions: aromatic hydrogens (H_r) from 6.0 to 8.0 ppm; H attached to oxygen groups in carbon ($H_{C–O}$) from 4.2 to 3.0 ppm, and aliphatic H (Ha) from 3.0 to 0.5 ppm. In accordance with results obtained by Wilson et al. (1983), the $H_{C–O}$ region was attributed to protons largely arising from polysaccharides. Several research groups have demonstrated that the Ha region might be affected by the presence of protons attached to aromatic rings in α β γ positions (Wilson et al., 1983, Gil–Sotres et al., 1994, and Simpson et al., 1997).

GC-MS Analysis Methylated LMr or HMr humic fractions were prepared by treatment with acetyl chloride in methanol suspension. Humic fractions (30 mg dry weight) and methanol (7 ml) were mixed in a 100-ml flask in an ice bath. One ml of acetyl chloride was slowly added to the resulting suspensions. Flasks were shaken for 4 hr at 25°C. This procedure was repeated three times, except that the last reaction was carried out overnight. Suspensions were centrifuged for 30 min at 2,680×*g*. The clear brown solutions were dried in a rotary evaporator and dissolved in 10 ml of methanol. The methylated humic fractions were injected into the GC-MS for IAA detection. All measurements were performed using an HP 6890 Gas Chromatograph (Hewlett Packard, Palo Alto, CA) coupled with an HP 5973 N Quadrupole (Hewlett Packard, Palo Alto, CA). Chromatographic separation of analytes was obtained with an HP 5MS capillary column with the following dimensions: length, 30 m; ID, 0.25 mm, and film thickness, 0.25 μm. The column had a constant helium flow of 0.7 ml min^{-1}, and the temperature regime was 1 min at 150°C followed by an increase of 10°C min^{-1} to 250°C, which was held for 15 min. The injector temperature was 250°C. All injections were performed in splitless conditions for 0.5 min. The mass spectrometer was in SCAN acquisition mode to detect the ions produced by electron ionization (70 eV) at the ion source temperature of 230°C, in the mass range from 50 to 350 Da, with a scan rate of 4.72 scan sec^{-1}. Pure IAA from Sigma (St. Louis) was methylated as described above for humic fractions and subjected to GC-MS. IAA detection in the chromatogram of methylated humic fractions was accomplished by comparing retention time and mass spectra with those of the purified methylated IAA sample.

^{13}C NMR Spectroscopy The relative intensity observed for a given chemical shift value, expressed as a fraction of the total signal intensity acquired, represents the proportion of that type of C present in the sample (Baldock and Preston, 1995). Spectra were divided into chemical shift regions assigned to chemical group classes aliphatic C (0–48 ppm), peptide and carbohydrate C (48–105 ppm), aromatic C (105–145 ppm), phenolic C (145–165 ppm), and carboxylic C (165–190 ppm). These assignments were based, in part, on values given

in Candler et al. (1997). By integrating the signal intensity contained within each chemical shift region, the proportion of a given type of C was calculated.

IAA Content Determination The quantitative determination of IAA in the humic fractions was made by an enzyme-linked immunosorbent assay (ELISA) (Phytodetek-IAA, Sigma, St Louis, MO). Tracer and standard solutions were prepared following the manufacturers' instructions. To each well, 100 μl of standard or LMr or HMr and 100 μl of diluted tracer were added. After an incubation at 4°C for 3 hr, the wells were decanted and washed by adding 200 μl of wash solution and then 200 μl of substrate solution. After 60 min at 37°C, 50 μl of stop reagent was added to each well, and the absorbance at 405 nm was then read using a 450 Biorad microplater reader (Biorad, Hercules, CA).

Fluorescent Labeling Indole-3-acetic acid (IAA) and humic substances (LMr and HMr) were conjugated with fluorescein isothiocyanate (FITC), according to the procedure described by Nairn (1969). A 2-ml sample (IAA or humic substances) of a 2% solution was added to 0.5 mg FITC completely dissolved in 1 ml of 0.1 M Na_2HPO_4, in a 25°C water bath. The pH of the solution was rapidly adjusted to 9.5 with drops of 0.1 M Na_3PO_4, and then, 0.145 M NaCl was added to make a total volume of 4 ml. The reaction was allowed to proceed for 30 min at 25°C and then stopped in an ice-water bath. Removal of unreacted fluorescent material was accomplished by gel filtration on a Sephadex G-15 (Pharmacia Biotech, Uppsala, Sweden) column. The fluorescent fractions were eluted and stored at −20°C until use.

Cell Culture Carrot cell suspensions were selected to provide a simple and rapid means of in vitro screening of compounds for activity before testing on whole plant systems.

Suspensions of *Daucus carota* cells (cv S Valery) were grown in liquid Gamborg's B5 medium (Gamborg et al., 1968), supplemented with sucrose (20 g l^{-1}), 2,4-D (0.5 mg l^{-1}), and 6-benzylaminopurine (6-BAP, 0.25 mg l^{-1}). Cultures were kept on a M 301-OR rotary shaker (Tecnovetro, Monza, Italy) at 100 rpm under 16 hr light/8 hr dark, at 25°C. Subculturing was carried out every 14 days, by inoculating each flask (containing 70 ml complete fresh medium) with 14 ml cell-suspension culture. After 14 days of growth, the cells were washed (3×'s) with B5 medium free of 2,4-D and 6-BAP, and resuspended in the same medium. Two ml of this suspension was inoculated into 18 ml of medium containing FITC–IAA (2 mg l^{-1}), FITC–LMr (0.2 mg C l^{-1}), or FITC–HMr humic fraction (1 mg C l^{-1}) and incubated for 14 days. The humic fraction concentrations in the range 0.1–5.0 mg C l^{-1} were chosen according to the results of previous experiments (data not published) that indicated these concentrations stimulated maximum cell suspension growth.

Microscopic Observations and Residual Fluorescein in the Culture Medium After different incubation times (3, 6, 12, 24, 48, 72, and 240 hr), cell suspensions were collected in glass tubes and centrifuged at 2,000×*g* for 15 min. The sedimented cells were observed by UV fluorescence microscopy (Zeiss Axiovert 135, Oberkochen, Germany) assessing the percentage of stained cells. An indirect, but global, estimation of fluorescein binding to cells was obtained by testing the residual fluorescein concentration in the different culture media. Supernatants were analyzed fluorometrically with a Perkin Elmer MPF 44 A spectrofluorometer (Perkin Elmer, Norwalk, CT); emission was recorded at 520 nm with an excitation wavelength of 495 nm. Fluorescence intensity was monitored on an arbitrary mV scale.

Blocking Tests Blocking test is a technique based on the competition between labeled and unlabeled substances for the same receptors or binding sites. Fluorescein staining of carrot cells was considered specific if binding of FITC was abolished by pretreatment with unfluoresceinated compound (whatever the identity of receptors) at the same concentration of the labeled preparations (blocking tests). Membrane cell staining is inhibited if unconjugated substance is adsorbed onto a cell membrane first. The incubation time was 6 hr, using a concentration of FITC ten times higher than that used in cell cultures.

To evaluate the possible interference of LMr humic substances with IAA binding to cell membranes, we carried out competitive blocking tests by treating samples of carrot cells with unconjugated LMr before incubation with FITC–IAA or *vice versa*. Microscopic preparation of carrot cells was done without use of fixatives to avoid histological or cytological distortion.

Results

^{1}H NMR Spectroscopy Distinct spectroscopic patterns were observed for the two humic fractions (Fig. 1). HMr showed an aromatic proton region and an intense and broad region (3.0–5.0 ppm) attributed to sugar-like and polyether components (Wilson et al., 1983; Wershaw, 1985). Inspecting the aliphatic region revealed an intense doublet at 1.33 ppm that was the result of protons of the β-CH_3 in lactate (Wilson et al., 1988; Fan, 1996; Francioso et al., 2000). Other broad and intense resonances at 0.83 ppm and 1.3 ppm are due to protons of the terminal methyl groups of methylene chains, respectively (Malcolm, 1990). Two further intense resonances appeared at 2.9 and 3.40 ppm and were assigned to protons in α-CH_3 of acetoacetate and ether aliphatics, respectively (Aldrich Library, 1992;

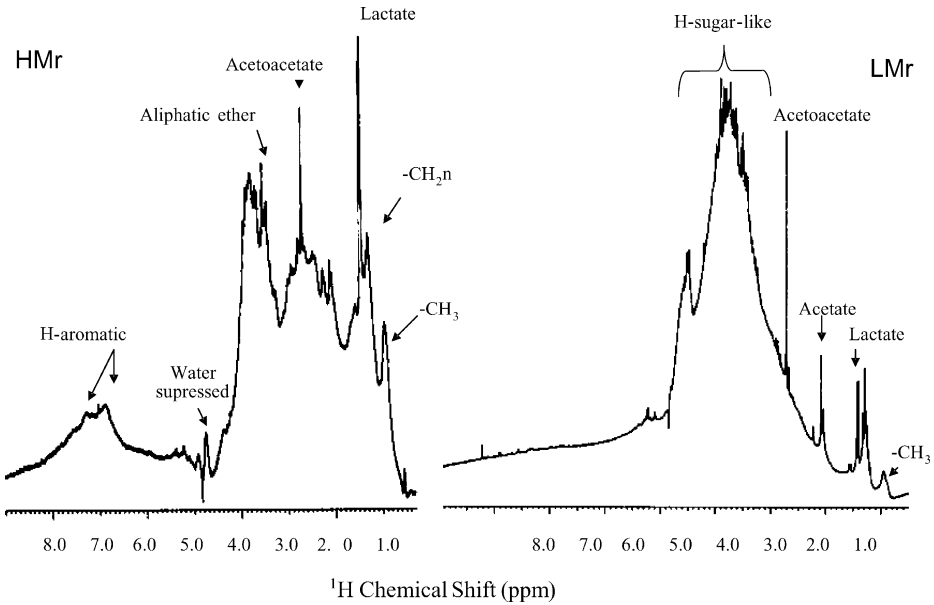


Fig. 1 ^{1}H NMR spectra (8.0–0.6 ppm) of humic substances extracted from the Ah horizon of a Calcaric Cambisol and separated in fractions corresponding to high (HMr) and low (LMr) relative molecular mass. The peaks are attributed according to Fan, 1996

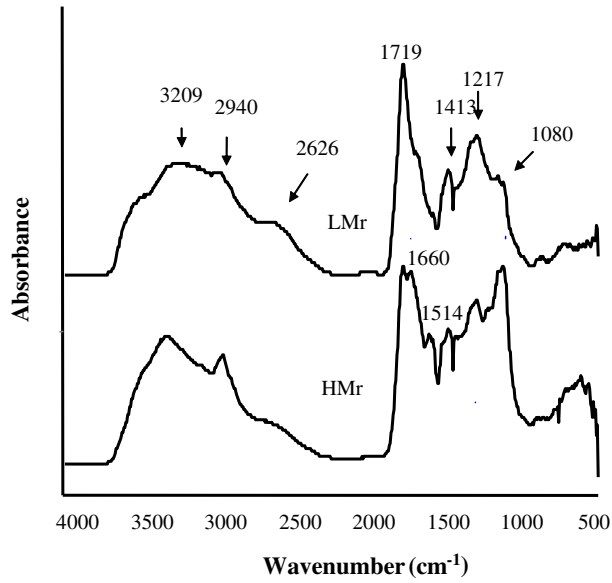


Fig. 2 DRIFT spectra of humic substances extracted from the Ah horizon of a Calcaric Cambisol and separated in high (HMr) and low (LMr) relative molecular mass fractions

Fan, 1996). In contrast, LMr did not show resonances due to aromatic protons and was characterized by a broad unresolved region, assigned to sugar-like components. However, the spectrum also contained some resonances due to the presence of low molecular weight organic substances, such as lactate (1.33 ppm), acetoacetate (2.9 ppm), and a singlet at 1.47 ppm, assigned to protons in β-CH_3 of alanine.

DRIFT Spectroscopy The interpretation of the DRIFT spectrum (Fig. 2) followed spectra published by Baes and Bloom (1989), Niemeyer et al. (1992), Stevenson (1994), and Francioso et al. (2001, 2002). The spectra of two humic fractions showed different structural modifications. In particular, in LMr appeared two strong bands at 1,719 and 1,217 cm^{-1} that are characteristic of C=O stretching (Rao, 1963; Niemeyer et al., 1992; Francioso et al., 1998, 2002) and C–O stretching motions in carboxyl acids, respectively. In addition, the broad band at 2,626 cm^{-1} suggested the formation of intermolecular hydrogen bonding between OH groups of COOH groups in acid dimers (Rao, 1963). In contrast, in HMr, the relative intensity of bands at 1,660 and 1,514 cm^{-1}, assigned to carbonyl C=O stretching and both N–H deformation and C–N stretching vibrations increased noticeably.

Table 1 Distribution of C intensity in different regions (ppm) of ^{13}C NMR spectra of high (HMr) and low (LMr) relative molecular mass humic fractions

Relative Intensity [Total Area Percentage (%)][a]					
Humic	0–48	48–105	105–145	145–165	165–190
Fraction	Aliphatic C	Peptidic and carbohydratic C	Aromatic C	Phenolic C	Carboxylic C
LMr	26.7±0.2	38.9±0.4	n.d.	n.d.	34.4±0.2
HMr	24.7±0.3	41.0±0.3	16.0±0.2	4.2±0.1	14.1±0.3

n.d. Not detected

[a] Values are mean ± SE (N=5)

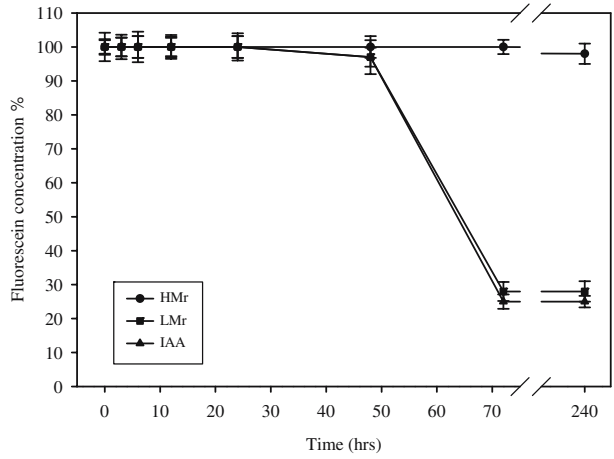


Fig. 3 Fluorescein concentration in solutions of carrot cells cultures supplemented with IAA, LMr, or HMr fractions

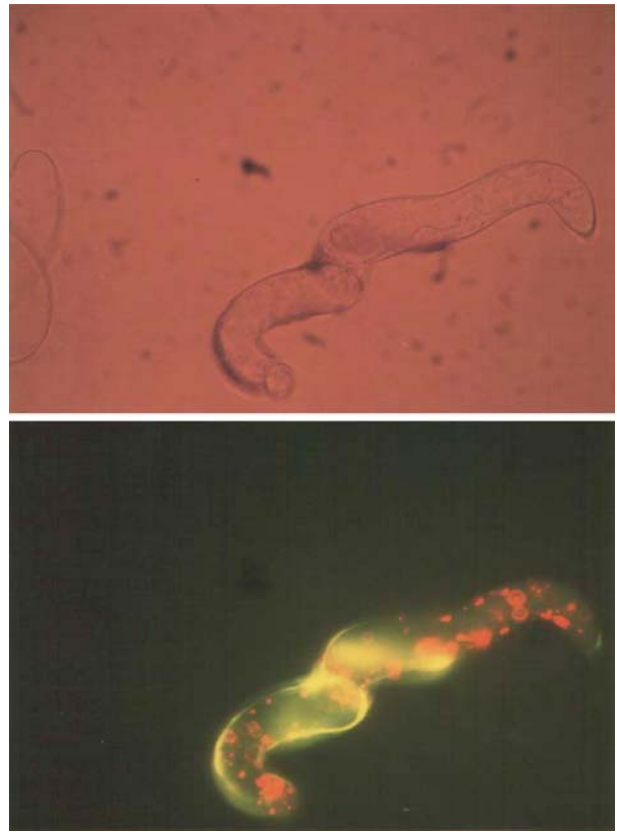


Fig. 4 Fluorescence labeling of bound IAA in carrot cell culture after 72 hr of incubation. In the carrot cells grown with FITC–IAA, 90% of cells were fluorescent after 72 hr of incubation. Fluorescence persisted through 240 hr

Moreover, these bands are due to C=C and C–C vibrations in aromatic rings, respectively. These results were supported by NMR spectra (Fig. 1).

GC-MS Analysis The IAA standard showed a GC retention time of 14.56 min and the mass spectrum had a base peak at 130 m/z, consistent with the ion formed by elimination of the $-CO_2$ Me radical from methylated IAA. This signal was not detectable in the spectrum for methylated humic fractions.

^{13}C NMR Spectroscopy Quantitatively, there were significant differences between spectra for the two humic fractions. The LMr humic fraction was richer in carboxylic and aliphatic C groups compared to the HMr fraction, whereas the HMr fraction had a higher content of peptidic-, carbohydratic-, aromatic-, and phenolic- groups (Table 1).

IAA Content The IAA content in the LMS and HMS fractions was determined to be 37 nmol mg^{-1} C.

Decrease in Fluorescence Intensity in B5 Medium Culture The fluorescence intensity was determined in the different experimental culture media after different incubation times (Fig. 3). At 72 hr, the fluorescein concentration in the culture media, added with FITC–IAA

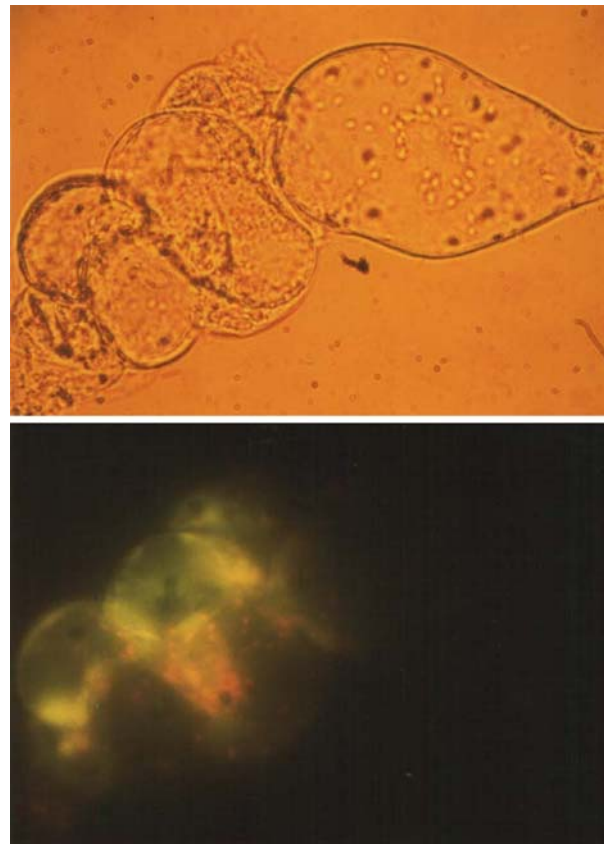


Fig. 5 Fluorescence labeling of bound LMr humic fraction in carrot cell culture after 72 hr of incubation. In the carrot cells grown with FITC–LMr humic fraction, 90% of cells were fluorescent after 72 hr of incubation and persisted through 240 hr

or FITC–LMr fractions, was markedly reduced, accounting in both about 30% of the baseline value. This decrease indicates an interaction of FITC compounds with carrot cells. On the other hand, the fluorescein content of the culture medium with HMr fraction was almost unchanged over 240 hr, suggesting a lack of interaction between this humic fraction and the carrot cells.

Microscopic Observations Cells grown in the presence of fluorescein-labeled IAA, LMr, and HMr were observed at different incubation times from 3 to 240 hr. Fluorescein staining, mainly of membranes, was observed in IAA and LMr treated cell cultures with a similar pattern. The staining was evident after 48 hr, but the highest fluorescence intensity, with more than 90% of carrot cells showing positive, was observed after 72 hr incubation, for both IAA and LMr treated cell cultures (Figs. 4 and 5). Membrane fluorescence persisted through 240 hr. Less than 10% of carrot cells grown in the presence of FITC–HMr were fluorescently stained at 72 hr, and this labile staining disappeared in cell specimens at longer incubation times (Fig. 6). Pretreatment with unconjugated IAA or LMr humic fraction blocked fluorescein staining of cells treated with both FITC–IAA (Fig. 7) or FITC–LMr humic fraction (Fig. 8). Blocking tests were considered positive if the number of fluorescent cells and the brightness of staining were decreased by 50% or more, with respect to the control samples.

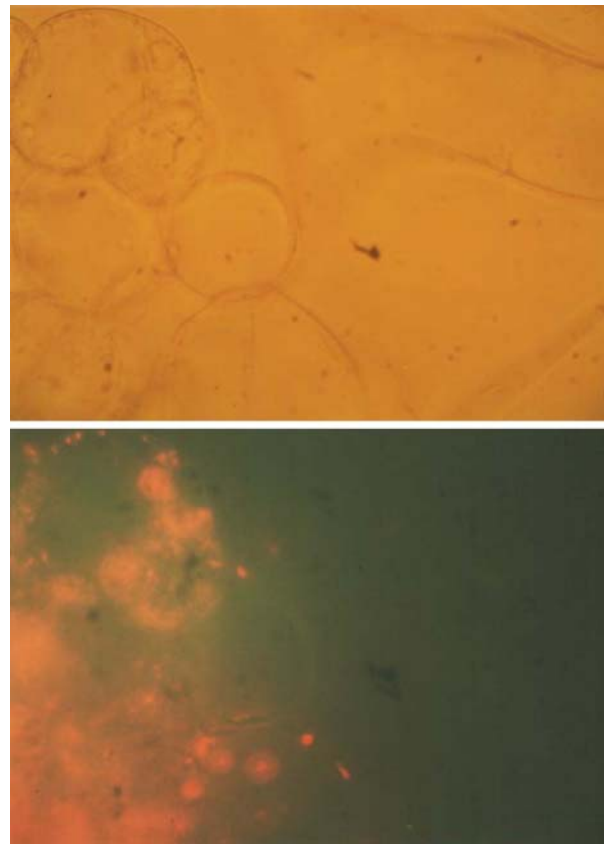


Fig. 6 Fluorescence labeling of bound HMr humic fraction in carrot cell culture after 72 hr of incubation. In the carrot cells grown with FITC–HMr humic fraction, less than 10% of carrot cells were fluorescently stained at 72 hr. This labile staining disappeared in cell specimens at longer incubation times

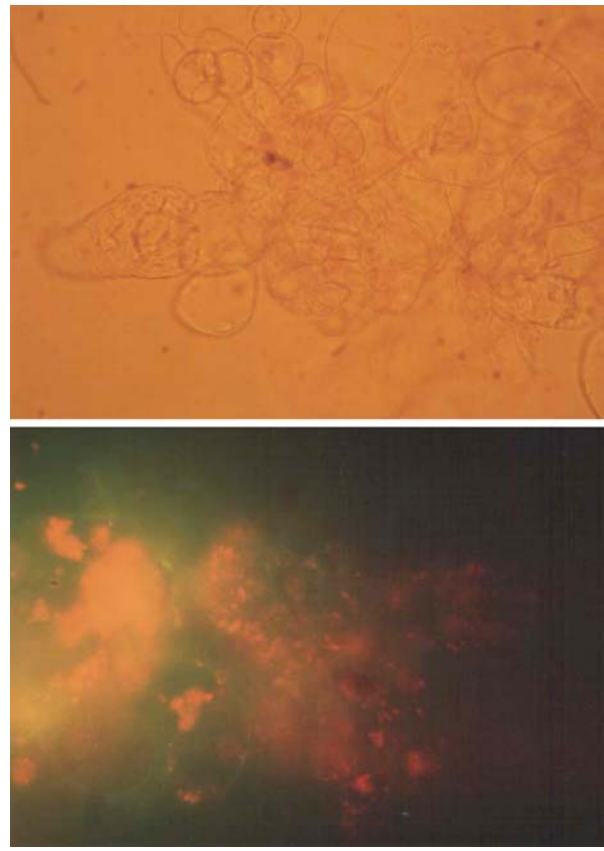


Fig. 7 Light and fluorescence microscopy of carrot cells pre-incubated with free IAA, to block auxin binding sites, and then, treated with FITC–LMr humic fraction. Pretreatment with free IAA blocked the fluorescence of carrot cell membranes. Granular staining is due to red auto-fluorescence

Discussion

Progress in research on HS has been considerably hampered by the lack of characterization of the humic fractions being used. A very detailed chemical characterization of the humic fractions used in this research had been undertaken by DRIFT, ^{1}H, ^{13}C NMR, and GC-MS. These substances were used to study their effects on carrot cell cultures and to evaluate their interaction with cell membranes.

In previous studies, the auxin-like biological effects of HS have been mainly attributed to the low relative molecular mass components of HS, such as LMr, in part, because of the ability of LMr to reach the plasma membrane of cells, whereas HMr is reported to be tightly bound to the cell wall (Vaughan, 1986). We have found marked differences in the structure of the two humic fractions. Comparing the DRIFT, ^{13}C, and ^{1}H MNR spectra of the two fractions revealed clear and significant differences. Quantitative ^{13}C NMR spectra showed the LMr fraction was rich in aliphatic- and carboxylic-C groups, whereas the HMr fraction had a higher content of peptidic-, carbohydratic-, aromatic-C, and phenolic-C groups. The higher content of aromatic groups in HMr was confirmed by ^{1}H NMR spectra that also

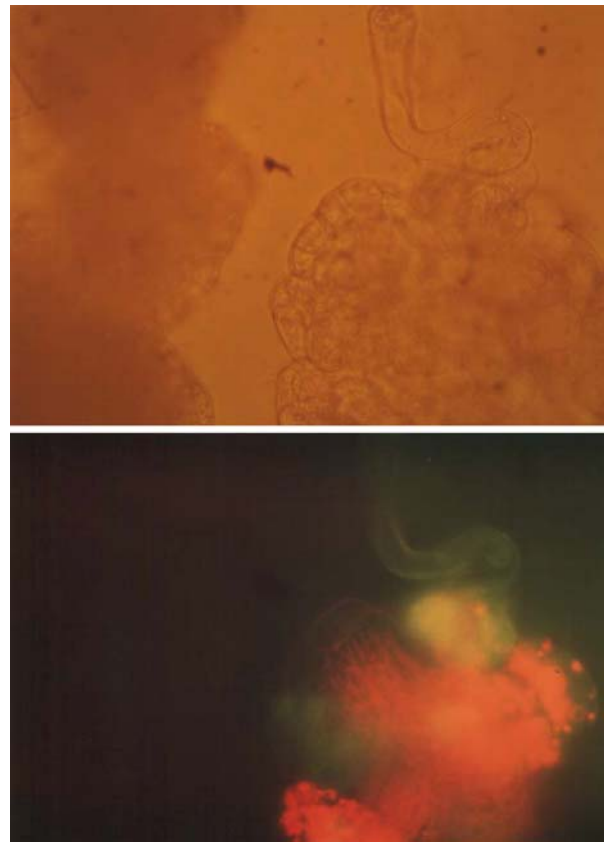


Fig. 8 Light and fluorescence microscopy of carrot cells pre-incubated with unconjugated LMr humic fraction and then treated with FITC–IAA. Pretreatment with unconjugated LMr humic fraction blocked the fluorescence of carrot cell membranes. Granular staining is due to red autofluorescence

suggested the presence of low molecular weight substances, such as lactate and acetoacetate, in both HMr and LMr. In HMr fraction, these substances are presumed to be bound to the macrostructure.

We demonstrate a direct binding of FITC–LMr humic fractions to cell membranes of *Daucus carota*. The morphological patterns and the intensity of fluorescent staining were similar to that observed in carrot cell cultures with FITC–IAA. The data obtained testing the decrease of fluorescein concentration in the media of cell cultures support these results; in fact, at 72 hr, there was good correlation between IAA or LMr interaction with plasma membrane and the maximum decrease of fluorescein concentration in the culture medium. The specificity of the binding is suggested by blocking test results. The blocking test is based on the competition between labeled and unlabeled substances for the same receptors or binding sites. Studies on the features of ABP1 showed an absence of change in conformation between auxin-bound and unbound (Woo et al., 2002), suggesting that the results obtained with the blocking test are not due to changes in conformation of the binding sites caused by the IAA pretreatment. Thus, the positive blocking competitive test between conjugated IAA and unconjugated LMr humic fraction or *vice versa* indicate that both substances interact in the same way with carrot cell membrane whatever the identity of auxin receptors is. These results cannot be related to the low content of IAA found in both fractions (0.5% w/w); in fact, it has been demonstrated that this amount of IAA is not able

to induce any biological actions on the vegetal systems utilized (entire plant, roots cells, and callus) (data not show). On the other hand, the FITC–HMr fraction, containing the same IAA amount (0.5% w/w) of the FITC–LMr fraction, did not show any interaction with the plasma membranes, and the fluorescein concentration in the culture medium did not change.

Several publications on the biological activity of humic material have shown the importance of the combination of a high content of both carboxyl, and phenolic groups, and low molecular size fractions (Muscolo et al., 1993, 1996; Nardi et al., 1994). The fraction with higher relative molecular mass was not effective on the biological activity of plant systems tested, but in soil, this fraction mainly contributes to the regulatory functions of humic fractions by encasing the strongly acidic LMr humic fraction in a macrostructure, thus, preserving it from leaching and degradation by microorganisms (Nardi et al., 1988). The dissociation of highly acidic and weakly acidic humic structures that takes place at the root–soil interface is, therefore, a prerequisite to any biological action of humic substances (Albuzio et al., 1993). Thus, the different interactions, and consequently, different biological activities of these humic fractions with the plant systems tested could be due to a different molecular mass associated with a different content of functional groups, in particular, the carboxylic groups that, as suggested by Rubery (1981) and Napier (2004), are needed for auxin bound.

In short, these results show that LMr and HMr humic fractions have a different interaction with carrot cell membranes, supporting the findings of previous studies of the effect of HS on plants, and in addition, it has been proven that the different biological activity of the two humic fractions tested is not due to the content of auxin, in that even though both humic fractions have the same amount of IAA, they do not induce the same biological activity.

Moreover, the interference in fluorescein staining of cell membranes between IAA and LMr humic fractions, as suggested by blocking competitive tests, suggests that IAA and LMr fractions richer in carboxylic groups, bind in the same way with carrot cell membranes, but do not give information about the structure of binding sites or the kind of bond because auxin specificity data have not yet been fully consistent with the characteristics for auxin receptors. Lately, affinity labeling has yielded a long list of proteins that do bind auxin, but for most (except for ABP1), their principal function is not a receptor (Napier et al., 2002, 2004); thus, many critical question about the molecular details and location of auxin binding sites remain to be answered (Woodward and Bartel, 2005).

Acknowledgments The authors thank Dr. Heather Macdonald for critical reading of the manuscript. This research was supported by the Università degli Studi "Mediterranea" di Reggio Calabria—Programmi di Ricerca Scientifica (ex Quota 60%).

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J Chem Ecol (2007) 33:131–146
DOI 10.1007/s10886-006-9200-2

Response to Host Volatiles by Native and Introduced Populations of *Dendroctonus valens* (Coleoptera: Curculionidae, Scolytinae) in North America and China

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Received: 18 May 2006 / Revised: 27 July 2006 / Accepted: 4 August 2006 /
Published online: 12 December 2006
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Abstract Bark beetles (Coleoptera: Curculionidae, Scolytinae) have specialized feeding habits, and commonly colonize only one or a few closely related host genera in their geographical ranges. The red turpentine beetle, *Dendroctonus valens* LeConte, has a broad geographic distribution in North America and exploits volatile cues from a wide variety of pines in selecting hosts. Semiochemicals have been investigated for *D. valens* in North America and in its introduced range in China, yielding apparent regional differences in response to various host volatiles. Testing volatiles as attractants for *D. valens* in its native and introduced ranges provides an opportunity to determine whether geographic separation

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promotes local adaptation to host compounds and to explore potential behavioral divergence in native and introduced regions. Furthermore, understanding the chemical ecology of host selection facilitates development of semiochemicals for monitoring and controlling bark beetles, especially during the process of expansion into new geographic ranges. We investigated the responses of *D. valens* to various monoterpenes across a wide range of sites across North America and one site in China, and used the resulting information to develop an optimal lure for monitoring populations of *D. valens* throughout its Holarctic range. Semiochemicals were selected based on previous work with *D. valens*: (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene, (*S*)-(+)-3-carene, a commercially available lure [1:1:1 ratio of (*R*)-(+)-α-pinene:(*S*)-(−)-β-pinene:(*S*)-(+)-3-carene], and a blank control. At the release rates used, (+)-3-carene was the most attractive monoterpene tested throughout the native range in North America and introduced range in China, confirming results from Chinese studies. In addition to reporting a more effective lure for *D. valens*, we present a straightforward statistical procedure for analysis of insect trap count data yielding cells with zero counts, an outcome that is common but makes the estimation of the variance with a Generalized Linear Model unreliable because of the variability/mean count dependency.

Keywords Bark beetles · Red turpentine beetle · Host attraction · Monoterpenes · Host–insect interactions · *Pinus* spp. · Invasive species · Upper confidence bound · Zero counts

Introduction

Olfactory cues play a crucial role in host location by insects, often mediating intra- and interspecific interactions among insects (Visser 1986; Bernays and Graham, 1988). Interactions between bark beetles (Coleoptera: Curculionidae, Scolytinae) and their conifer hosts are of interest because many species colonize and kill apparently healthy trees, resulting in significant economic losses (Wood 1982b). Many species have specialized feeding habits and attack one or a few closely related host genera (Sturgeon and Mitton, 1982; Kelley and Farrell, 1998). Volatiles released from trees can provide long- and/or short-range cues for beetle attraction and, in some cases, synergize beetle aggregation pheromones (Wood 1982a; Byers 1995; Miller and Borden, 2000; Erbilgin and Raffa, 2000).

The role of monoterpenes in the ecology of bark beetles is of interest because some, such as α-pinene and myrcene, are precursors of the aggregation pheromone components of several bark beetle species (Hughes 1974; Renwick et al., 1976; Hendry et al., 1980; Byers 1989). Other studies have demonstrated *de novo* synthesis of pheromones in some species (Seybold et al., 1995b; Ivarsson et al., 1997).

The red turpentine beetle, *Dendroctonus valens* LeConte, is widely distributed in North America, extending from Nova Scotia (Canada) west to California (USA) and south to Honduras, except for the southeastern United States where its sibling species, *D. terebrans* (Oliv.), occurs (Wood 1982b). Although it is a relatively minor pest within its native range, *D. valens* occasionally kills young ponderosa pines in plantations in the western USA (Eaton and Rodríguez Lara, 1967; Rappaport et al., 2001) and may predispose trees to subsequent lethal attacks by other species (Klepzig et al., 1996; Owen et al., 2005). Drought-stressed trees appear to be more susceptible to attack by *D. valens* (Smith 1961), and there is concern that global climate change may exacerbate damage caused by this insect. Larvae feed and develop in the phloem of host trees, and galleries often overlap spatially and temporally with larvae of other members of the subcortical-feeding guild of

insects (Wood 1982b; Erbilgin and Raffa, 2002). *D. valens* was introduced into China around 1985, and has caused extensive mortality of *Pinus tabuliformis* Carrière and other pine species in north central China (Li et al., 2001). Although *D. valens* has widely been considered a secondary pest in North America (Furniss and Carolin, 1977), it has been shown to act as a primary tree-killer in China. The reasons underlying the greater tree mortality in China are not known, but could include a lack of evolutionary adaptation with this new insect/host association (Sakai et al., 2001) and/or differences in host physiology, stand structure, or climate. Considering its wide host range and geographic distribution in North America and the abundance of suitable pine hosts throughout Eurasia (Critchfield and Little, 1966), the host range and distribution of *D. valens* in the Palearctic region are expected to increase.

D. valens exploits volatile pine monoterpenes as kairomones to locate host trees, and several monoterpenes have been tested for attraction of *D. valens* in both North America and China, revealing different responses in populations from different regions. Hobson et al. (1993) reported that traps baited with (+)-α-pinene, (+)-3-carene, and (−)-β-pinene were the most attractive monoterpenes tested in California, with (−)-β-pinene by far the most attractive for *D. valens*. They showed a positive dosage–response relationship in tests of (−)-β-pinene at high release rates. Fettig et al. (2004) showed positive response by *D. valens* to the standard three-component commercial lure consisting of a 1:1:1 ratio of (*R*)-(+)-α-pinene:(*S*)-(−)-β-pinene:(*S*)-(+)-3-carene (Phero Tech, Inc., Delta, BC, Canada) in California populations, but they did not test individual monoterpenes for relative attraction. Erbilgin and Raffa (2000) showed attraction of *D. valens* to (+)-3-carene and both enantiomers of α-pinene in Wisconsin. In China, (+)-3-carene was the most attractive monoterpene for *D. valens*, with beetles showing a dose-dependent increase in attraction to (+)-3-carene, but not to (−)-β-pinene (Sun et al., 2004).

These differences were puzzling, but it was not possible to make reasonable inferences about geographic variation in response because test conditions, notably release rates, varied widely among these earlier studies. Studies conducted in California, for example, used release rates several hundreds of times higher than those in China (Hobson et al., 1993; Sun et al., 2004). Other semiochemicals cause dose-dependent differences in insect behavior (Miller and Borden, 2000), so it is reasonable to question whether the behavioral differences reported in earlier studies resulted from differences in release rate rather than divergence in behavioral response of the Chinese populations from the indigenous North American populations. On the other hand, other widespread insect species use semiochemical "dialects" in disparate parts of their ranges (Wood 1982a; Seybold et al., 1995a; Erbilgin et al., 2001; Miller et al., 1997), so we could not rule out geographic variation in behavioral response to host monoterpenes by *D. valens*, especially considering the localized source of populations introduced into China from the Pacific Northwestern United States (Cognato et al., 2005). These apparent differences prompted us to conduct a systematic evaluation of *D. valens* response to host monoterpenes throughout its native range in North America and its introduced range in China, by using standardized (i.e., with equivalent release rate) lures containing individual host monoterpenes. These were compared with a three-component commercial lure used widely in North America.

Testing host volatiles as attractants for species with wide host and geographical ranges provides us with an opportunity to compare semiochemical-mediated host selection behavior in native and introduced ranges. Furthermore, understanding the ecological aspects of host selection is important for developing semiochemicals to monitor beetles (Wood 1982a; Schlyter and Birgersson, 1999). Our objective was to investigate the responses of *D. valens* to various monoterpenes across a wide range of sites across North

America and China and to assess the potential behavioral divergence of the populations. Our practical goal was to develop optimal blends or single-component lures, appropriate for each locality, for monitoring populations of *D. valens* throughout its Holarctic range.

The project, involving nine separate trapping studies, also provided an opportunity to describe an approach for estimating treatment differences in data sets with many "zero count" cells. Data sets with zero count cells have long been a problematic area for entomological researchers (Reeve and Strom, 2004) because it is not possible to estimate the standard errors of cells with no observations. We can, however, calculate theoretical confidence intervals for the expected means for these cells, allowing Bonferroni-adjusted comparisons (Miller 1991) between means from zero count cells and means from cells with nonzero observations. This problem has been addressed in the past by either recommending exclusion of zero-count cells from the analysis, adding a small constant to each count, or creating a "dummy variable" with a low mean and variance (Agresti 1996; Reeve and Strom, 2004). In contrast, our approach was to calculate Bonferroni-adjusted confidence intervals for means with zero observations to permit comparison with nonzero cells with a given level of certainty, thus using real data rather than "dummy" variables.

Methods and Materials

Study Sites The experiments were conducted in 2003, 2004, and 2005 at 6 sites spanning the range of *D. valens* in North America and at one site in China. These were in the following forests: a *P. resinosa* Ait. stand in Jackson Co. (Central WI, USA); an east side *P. ponderosae* Laws plantation in Siskiyou Co. (Northern CA, USA); a Sierra mixed conifer forest where *D. valens* colonizes *P. lambertiana* Dougl and *P. ponderosa*, in Blodgett Forest Research Station (Central CA, USA); a mixed conifer forest where *D. valens* colonizes *P. jeffreyi* Grev. & Balf. and *P. coulteri* D. Don, in the Laguna Mountains in San Diego Co. (Southern CA, USA); a mixed hardwood and conifer forest where *D. valens* colonizes *P. strobus* L. and *P. resinosa* at Raccoon Creek State Park in Beaver Co. (Western PA, USA), a natural *P. leiophylla* Schl. et Cham. stand in San Francisco Zentlalpan (Estado de México, México), and a *P. tabuliformis* plantation in Shanxi Province, China.

Experimental Design We selected semiochemicals based on previous work with *D. valens* (Hobson et al., 1993; Erbilgin and Raffa, 2000; Sun et al., 2004). Four single-monoterpene baits [(*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene, and (*S*)-(+)-3-carene], the standard commercial red turpentine beetle lure, hereafter referred to as the "RTB lure," which consists of a 1:1:1 ratio of [(*R*)-(+)-α-pinene:(*S*)-(−)-β-pinene:(*S*)-(+)-3-carene; Phero Tech Inc.], and a blank control were replicated at each site (Table 1). Trapped beetles were collected periodically, but the number of collections ("sampling periods" in Table 1) varied from site to site for reasons of local logistics. The number of replicates at each site was constant throughout the entire sampling period for each study. All of the analytical models accommodated these repeated measures, and in cases where the number of sampling periods was small (e.g., Pennsylvania), the number of replicates was increased to reduce the effect of overdispersion of counts.

Chemicals (all with purity >95%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and from Phero Tech. Single-monoterpene release devices consisted of 15-ml polyethylene bottles (Phero Tech), each filled with 8 ml of the monoterpene. The high cost of enantiopure monoterpenes prohibited the use of the full measure of 15 ml per vial. To

Table 1 Site locations and sampling design for testing attraction of *Dendroctonus valens* to pine monoterpenes

Site	Number of Replications	No Sampling Periods
Northern California	5	5
Central California	7	6
Southern California		
2003	5	2
2004	5	5
Wisconsin	6	3
Pennsylvania	10	2
Mexico	8 (4 for control)	1
China		
2003	5	7
2004	10	19

ensure that the entire contents of each vial were exposed to the release surface, the bottles were squeezed until the liquid reached the shoulder, then were tightly capped leaving the bottles slightly collapsed. Each bottle also contained 100 mg BHT (butylhydroxytoluene) (Sigma-Aldrich) as an antioxidant. Each chemical maintained a sustained release of 103.4± 11.3 mg/d at 20±4.3°C with 6.2±2.3 km/hr wind velocity for at least 30 d in the field. Release rates of chemicals (determined by daily weight loss) under the same conditions did not vary significantly ($F_{4,3}$=1.23, P=0.45). The RTB lure contains 15 ml of the three-component blend (5 ml of each of the three monoterpenes) mixed with undefined stabilizers and antioxidants, and it releases at a slightly higher rate, 110 mg/d, under similar environmental conditions (personal communication, B. Thompson, Phero Tech). We concluded that this slight difference in release rate would have no significant effect on our overall inferences, especially considering the ambient release of monoterpenes from trees in a typical forest stand (Wilt et al., 1993; Schade and Goldstein, 2003).

Lures were attached to multiple-funnel traps (Phero Tech) or panel traps (Advanced Pheromone Technologies, Marylhurst, OR, USA) designed for trapping bark beetles. Propylene glycol antifreeze or a plastic insecticidal strip saturated with 2,2-dichlorovinyl dimethyl phosphate (Hercon Vaportape II Insecticidal strips; Hercon Environmental Co., Emigsville, PA, USA) was put in each collection cup to prevent predation. The experimental design was a completely randomized design with repeated measurements, with the exception of the Wisconsin site, which was a randomized complete block design. For some sites, traps were rerandomized at sampling time, and for other sites the trap was left at the same location for the entire time of the experiment. Statistical models for analysis were designed to account for these minor differences in design. The numbers of replications and sampling periods per treatment are shown in Table 1. Beetles captured in each trap were identified, counted, and recorded; voucher specimens were stored at the USDA Forest Service Institute of Forest Genetics (Placerville, CA, USA), Universidad Autónoma Chapingo (Texcoco, México), or Chinese Academy of Sciences (Beijing, China) according to site.

Statistical Analyses Trap counts were analyzed with Poisson Regression Models for overdispersed Poisson distributed responses (counts) to address potential overdispersion arising from the repeated measurements (McCulloch and Searle, 2001). The overdispersion error was assumed to be normally distributed for some sites according to sampling design.

The Maximum Likelihood Ratio test with the Bonferroni adjustment was used for multiple comparisons for an experimentwise error rate of 0.05. The SAS (2005) NLMIXED procedure was used to estimate parameters and their differences. Mean counts for treatments with nonzero observations were estimated the using the computing routines described above. For the treatments with zero observations, the mean counts were estimated as zero, and precision was estimated by a Bonferroni-adjusted confidence interval from 0.0% to 99.7% $(100 \times [1 - 0.05/\{15 = \text{number of comparisons}\}])$, where the upper confidence bound was calculated by using rules for multiplication and addition of probabilities (Hoel et al., 1971). We thereby created a way to determine, with a given level of certainty, the range of the treatment means with zero observations. We compared nonzero count treatment means by using the standard method of testing significance, but for zero-count treatment means we used the overlap between the calculated Bonferroni-adjusted confidence intervals for zero counts and the Bonferroni-adjusted confidence intervals of the nonzero treatment means. Even though this approach is occasionally conservative, it yields a more sensitive test of treatment differences. In general, it works best if the lower confidence bounds of the nonzero count treatment levels are not close to zero.

Procedure for Calculating the Upper Bound for Treatments with Observed Zero Counts Upper bounds are developed as follows. We present a $1 - \alpha$ upper bound for three count distributions: the Poisson distribution and two overdispersed Poisson distributions (Poisson-Gamma = Negative Binomial and Poisson-Normal).

Let α be the given level; therefore, a $1 - \alpha$ upper bound for expected count for a treatment is given by:

$$P(x > 0) = 1 - [P(x = 0)]^{n_{\text{reps}}} \leq 1 - \alpha,$$

where n_{reps} is the number of replications.

(A) Suppose x has the Poisson distribution with mean λ_T (not overdispersed) for a given treatment level T, then

$$P(x > 0) = 1 - \left[e^{-\lambda_T}\right]^{n_{\text{reps}}} \leq 1 - \alpha.$$

Therefore, the upper bound for expected mean count is given by

$$\lambda_T \leq -\frac{1}{n_{\text{reps}}} \log(\alpha), \tag{1}$$

where log indicates logarithm.

The next two techniques [Eqs. (2) and (3)], which assume the Poisson-Gamma or the Poisson-Normal distribution, can be problematic to calculate if the software is not available. If so, then a close approximation of the upper bound can be made by using just the Poisson distribution [Eq. (1)], since the α level and number of replicates are the only pieces of information required for that calculation.

(B) Suppose x has an overdispersed Poisson distribution with mean λ_T, and with a Gamma distributed overdispersion error (McCulloch and Searle, 2001). Therefore, x has the Negative Binomial distribution (k). In this case,

$$P(x > 0) = 1 - \left[(1 - k\lambda_T)^{-\frac{1}{k}}\right]^{n_{\text{reps}}} \leq 1 - \alpha,$$

and the upper bound is given by

$$\lambda_T \leq \frac{\alpha^{\frac{-k}{n_{\text{reps}}}} - 1}{k} \tag{2}$$

The parameter k can be estimated by SAS NLMixed or Glimmix procedures.

(C) Suppose, for a given treatment, x has an overdispersed Poisson distribution $P(\lambda_T)$ with mean $\lambda_T = e^{T+\sigma\varepsilon}$, and a normally distributed overdispersion error ε (McCulloch and Searle, 2001.). If ε is $N(0,1)$ (Standard Normal) distributed, and σ is the standard deviation of the overdispersion error, then

$$P(x>0) = 1 - \left[\int_{-\infty}^{+\infty} \frac{e^{-e^{T+\sigma\varepsilon}} e^{-\frac{\varepsilon^2}{2}}}{\sqrt{2\pi}} d\varepsilon\right]^{n_{\text{reps}}} \leq 1-\alpha,$$

where $\int$ is the integration symbol. In this case, the upper bound does not have a closed formula. However, it can be approximated by numerically calculating this probability for several values of T for the following function:

$$f(T) = \left[\int_{-\infty}^{+\infty} \frac{e^{-e^{T+\sigma\varepsilon}} e^{-\frac{\varepsilon^2}{2}}}{\sqrt{2\pi}} d\varepsilon\right]^{n_{\text{reps}}} \tag{3}$$

The numerical solution of the integral can be done with Mathematica (Wolfram 1999). Fig. 1 depicts the function $f(T)$ for possible upper bound values for several values of T. The value of T for a given $f(T)=0.05/15=0.0033$ is equal to 1.098 (the upper 99.7% confidence limit). Fig. 1 diagrams the method for the Central California site; the upper bound for the treatment levels with zero counts for this site can be compared with the lower bounds of other treatments at the same α level ($\alpha=0.0033$).

Panels A–D, F, and G of Fig. 2, each of which contains at least one zero-count cell, all illustrate this approach for making multiple comparisons with treatments with zero counts (the upper Bonferroni-adjusted confidence limit for multiple comparisons is indicated by a dotted line on those figures). The calculated upper bound for the treatment levels with zero counts can be compared to the lower bounds of other treatments at the same α level ($\alpha=$

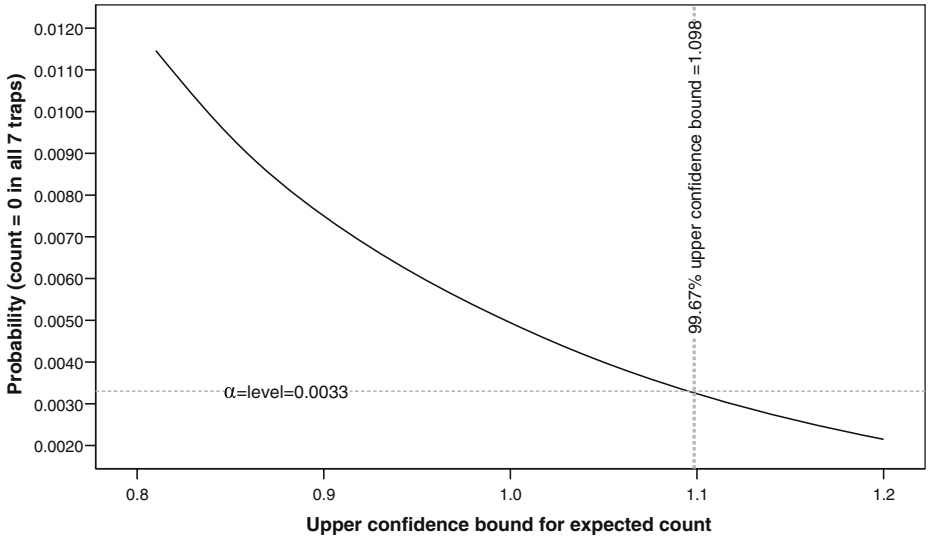
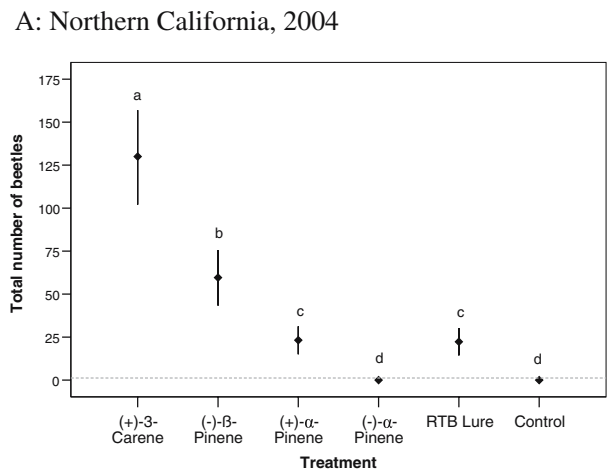
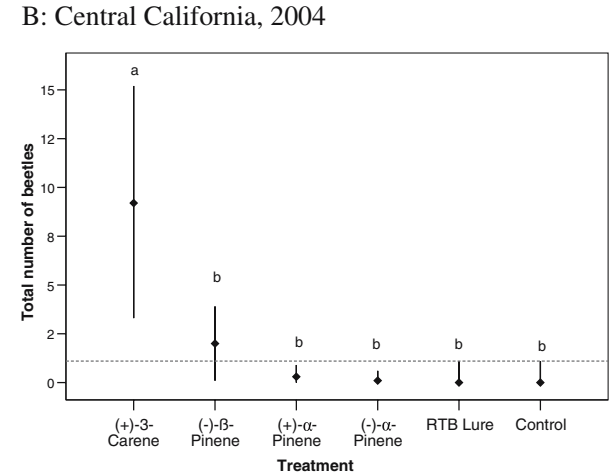


Fig. 1 Calculated upper Bonferonni-adjusted confidence limit for cells with zero counts (example using results from central California, 2004)





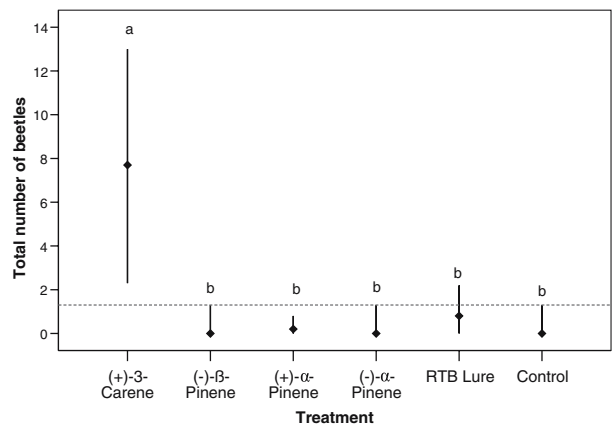
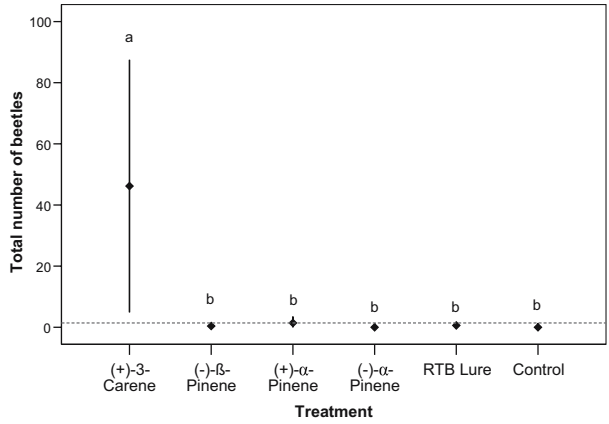


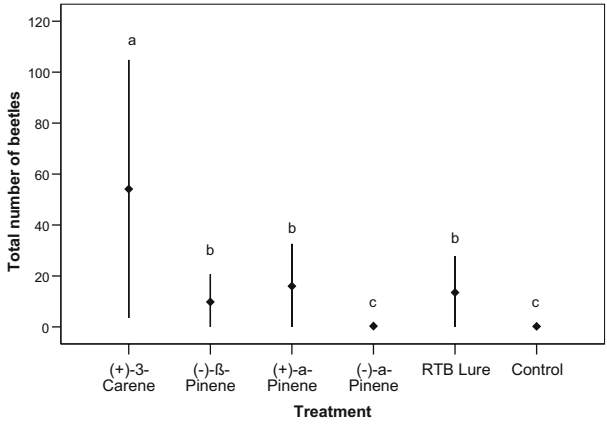
Fig. 2 (A-I) *Dendroctonus valens* response to pine monoterpenes at nine different localities and/or years in North America and China. Dates (location codes) for experiments: May 22–June 10 for Northern California (CA North); September 21–October 10 for Central California (CA Central); August 28–September 6 and May 20–August 26 for Southern California (CA South); May 3–May 18 for Wisconsin (WI); May 2–May 15 for Pennsylvania (PA); June 27 for Mexico (MX); May 2–June 20 for China 2003 (CH 2003) and May 4–August 2 for China 2004 (CH 2004). Data show mean numbers (lower=99.7% lower confidence bound, upper=99.7% upper confidence bound) of insects per trap summed over sampling period. Means followed by the same letter in a column are not significantly different from each other. Dotted lines indicate 99.7% calculated upper confidence bound for treatments with zero observations

Fig. 2 (Continued)

D: Southern California, 2004



E: Wisconsin, 2004



F: Pennsylvania, 2004

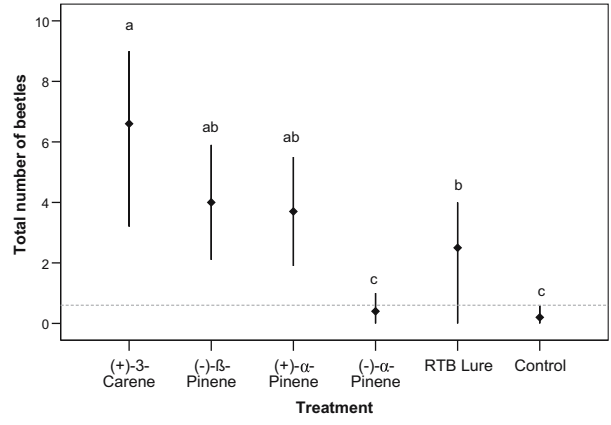
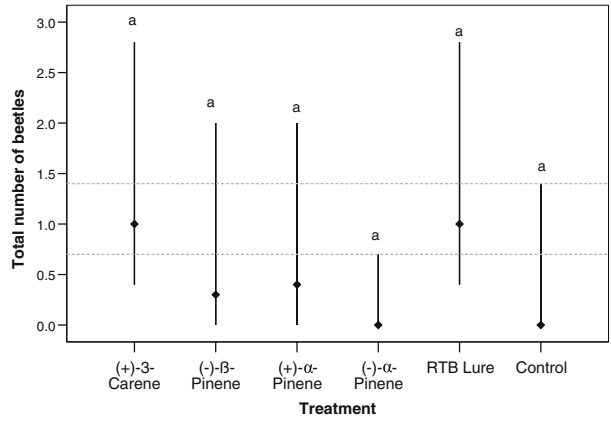
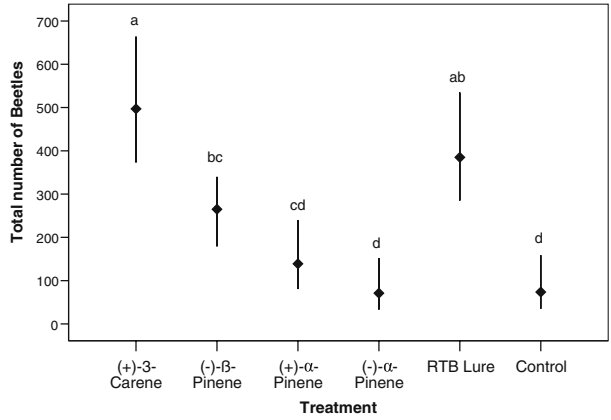


Fig. 2 (Continued)

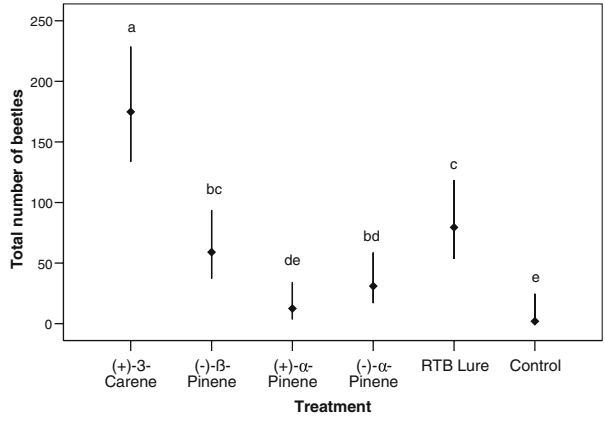
G: Mexico, 2005



H: China, 2003



I: China, 2004



0.0033). If they do not touch, then the treatment levels are significantly different. If they overlap, then there is not enough evidence to reject the null hypothesis. Mexico has two calculated upper bounds because each treatment level had eight replications except for the control, which had only four (and thus a greater upper bound). Fig. 2E, H, and I, which do not contain zero count cells, are handled in the traditional manner.

Results

Trap counts were estimated as mean numbers of beetles per trap summed over the sampling intervals (Table 1, Fig. 2A–I). The mean counts of beetles summed over sampling intervals are not comparable among sites because the sites had different sampling time ranges, from 1 wk for Mexico to nearly 3 mo for China and southern California in 2004 (Table 1).

California In northern California (Fig. 2A), (+)-3-carene was the most attractive compound tested, followed by (−)-β-pinene. The number of *D. valens* trapped was not different between (+)-α-pinene and the RTB lure, nor between (−)-α-pinene and the control. In central California (Fig. 2B), the numbers of *D. valens* were relatively lower than those in northern California, but the pattern of attraction was similar; only (+)-3-carene attracted significantly more *D. valens* than the control. In southern California (Fig. 2C and D), the results were identical to those of central California. In two consecutive years, only (+)-3-carene attracted a significantly higher number of *D. valens* than the control.

Wisconsin (+)-3-Carene was significantly more attractive than the remaining treatments, whereas (+)-α-pinene, (−)-β-pinene, and the RTB lure were more attractive than (−)-α-pinene and the control (Fig. 2E). There were no significant differences between mean numbers of *D. valens* caught in traps baited with (−)-α-pinene and the control, nor between (+)-α-pinene, (−)-β-pinene, and the RTB lure.

Pennsylvania (+)-3-Carene was the most attractive compound tested and was significantly different from the control, the (−)-α-pinene, and the RTB lures (Fig. 2F). Attraction of (−)-β-pinene and (+)-α-pinene were not different, and both were greater than the attraction of either (−)-α-pinene or the control.

Mexico None of the treatments in Mexico were significantly different from one another (Fig. 2G). However, there was only one sampling interval, and the number of beetles was low, with a median close to zero for all treatments except for (+)-3-carene and the RTB lure.

China (+)-3-Carene was the most attractive lure in both 2003 (Fig. 2H) and 2004 (Fig. 2I), but only in 2004 was it significantly more attractive than the RTB lure, which was the second most attractive lure in both years of testing. The mean number of *D. valens* caught by the RTB lure was not significantly different from that caught by (−)-β-pinene in both 2003 and 2004. The mean number of *D. valens* caught in (−)-β-pinene traps was significantly higher than for the control. (+)-α-Pinene, (−)-α-pinene, and the control were not significantly different from one another in 2003. Results for 2004 were identical except that (−)-α-pinene caught more insects than the control.

General Observations With the exception of the tests conducted in Wisconsin (Fig. 2E) and China (Fig. 2H and I), where beetle populations were among the highest, the total number of beetles trapped in unbaited traps was zero. There were significant differences among treatments in the mean numbers of *D. valens* caught in all sites and all years except for Mexico (2005), where beetle numbers were low and there was only one sampling interval (Fig. 2G). (+)-3-Carene was the most attractive treatment at all sites, and the difference between (+)-3-carene and the second most attractive treatment was significant in most cases, i.e., northern California (Fig. 2A), central California (Fig. 2B), southern California (both years) (Fig. 2C and D), Wisconsin (Fig. 2E), and China (both years) (Fig. 2H and I). In Pennsylvania (Fig. 2F), (+)-3-carene attracted 65% more beetles than did (−)-β-pinene, but this difference was not significant. In Mexico (Fig. 2G), (+)-3-carene tied with the RTB lure for most attractive treatment. The RTB lure, which also contains (+)-3-carene as a component, was generally the second most attractive lure, although in most cases it was not significantly more attractive than the third-ranking treatment. In three cases, however (northern California, central California, and Pennsylvania), (−)-β-pinene was the second most attractive compound tested, but only in northern California was this difference significant. In Wisconsin, (+)-α-pinene was the second most attractive treatment but it was not significantly different from (−)-β-pinene or the RTB lure. (−)-α-Pinene was not significantly different from the control in any case except for China in 2004.

Discussion

(+)-3-Carene was the most attractive monoterpene tested throughout the native range of *D. valens* and in China. These results confirm studies conducted recently in China, where (+)-3-carene has been shown to be the dominant attractant among pine host monoterpenes (Sun et al., 2004; J.H. Sun, personal communication). (+)-3-Carene is, thus, broadly applicable for monitoring *D. valens* in both China and North America. This uniform preference for the same signal over such a broad geographical range differs from responses by certain other bark beetle species to their pheromones, which vary geographically (Wood 1982a; Seybold et al., 1995a; Miller et al., 1997).

The practical, pest management implication of this finding is clear: a single-component lure will likely be the most cost-effective and biologically efficient one for *D. valens* across its range in the Holarctic, whether in its native range in North America or in its introduced range in Asia. Preferential attraction of *D. valens* to (+)-3-carene suggests that it is the key stimulus from its host trees, and plays role in host selection. Differences between this and earlier studies (Hobson et al., 1993; Erbilgin and Raffa, 2000) may result from differences in release rates of the compounds. For example, in the work of Hobson et al. (1993), where (+)-3-carene was less attractive than other monoterpenes, release rates were more than 200 times those used in this study. In other words, (+)-3-carene may become repellent or inhibitory at higher release rates relative to the other monoterpenes studied.

(+)-3-Carene constitutes varying proportions of the resins of *Pinus* species and other coniferous genera in North America (Mirov 1961; Hobson et al., 1993; Smith 2000; Erbilgin et al., 2001), but it is generally the predominant monoterpene in its most widely distributed host in North America, ponderosa pine (Smith 2000). *P. tabuliformis*, the primary host of *D. valens* in China, has a needle monoterpene composition that is similarly high in (+)-3-carene (Jin et al., 1994). Several other forest Coleoptera respond to 3-carene as an attractant or synergist. For example, the pine shoot beetle, *Tomicus piniperda*, utilizes

(+)-3-carene as a kairomone along with (±)-α-pinene and terpinolene to locate and assess the susceptibility of its host, *P. sylvestris* L. (Byers et al., 1985; Lanne et al., 1987; Byers 1992). Likewise, *Pityogenes chalcographus* (L.) utilizes α-pinene, β-pinene, and (+)-3-carene to locate and colonize hosts (Byers et al., 1988). In another study, (+)-3-carene synergized the attraction of *Conophthorus ponderosae* Hopkins to its sex pheromone in a ponderosa pine seed orchard in northern California (Gillette et al., unpublished data). (+)-3-Carene also influenced the attraction of bark beetles and their predators to bark beetle pheromones (Miller and Borden, 2003).

Although host monoterpenes are crucial in the *D. valens* host selection process, it is difficult to relate levels of emission from our lures to natural emissions from forest stands. For example, monoterpene concentrations in pines vary in response to drought (Lorio et al., 1995), fungal infection of roots (Klepzig et al., 1995), and defoliation (Wallin and Raffa, 1999). Emissions from foliage vary depending on state of decomposition (Wilt et al., 1993). Furthermore, boring activities initiated by beetles rapidly alter monoterpene concentrations and release rates of attacked trees (Paine and Stephen, 1987; Cook and Hain, 1988; Raffa and Smalley, 1995). On a broader scale, estimations of volatile emissions from forests are more difficult to make. For example, natural emission of α-pinene from ponderosa pine plantations at the Blodgett Forest Research Station is about 0.1 $mg/m^2/hr$ during the daytime in summer, and it changes as an exponential function of temperature (Schade and Goldstein, 2003). Monoterpene emissions from the same pine forest were enhanced about 40 times during a mechanical thinning operation. Previous reports indicate that bark beetle populations tend to increase following fire and tree thinning (Furniss and Carolin, 1977), and higher emissions of monoterpenes may explain such increases in populations of *D. valens* and other bark beetle species.

The broad host and geographical range (Kelley and Farrell, 1998), highly specialized olfactory responses (White and Hobson, 1993), and cryptic nature (Bernays and Graham, 1988) of *D. valens* may all have contributed to its successful establishment in China. Other factors, such as host susceptibility and a lack of competition and natural enemies (Sakai et al., 2001), undoubtedly also played a role. Although many species of *Dendroctonus* are restricted to one of four confamilial genera of conifers (Sturgeon and Mitton, 1982), *D. valens* can colonize almost three times as many hosts as its congeners (Kelley and Farrell, 1998), suggesting that it may have greater intrinsic capacity for adaptation to new hosts. Recently, Cognato et al. (2005) reported significant haplotype variation in *D. valens* in its native and introduced ranges. We initially expected that behavioral differences seen in earlier studies might be explained by genetic variability, but this variation was not reflected in behavioral differences in responses to host compounds.

The recent introduction of *D. valens* to China and its rapid establishment on pines indigenous to Asia demonstrate that *D. valens* can colonize and reproduce on new host species within the same genus as its ancestral hosts in North America. This new association may have resulted from adaptation to diverse secondary chemicals during colonization of ancestral hosts (Byers 1995; Kelley and Farrell, 1998), and/or close association with particular host volatiles that result in gaining access to chemically similar host species in new biogeographic areas (Futuyma et al., 1995; Becerra 1997). Recognition of common host volatiles, e.g., (+)-3-carene, may be the first phase of colonization of new host species by *D. valens*, since the dominant volatiles of its hosts are mainly monoterpenoids (Mirov 1961; Juuti et al., 1990; Smith 2000). However, the presence of the same attractive compounds in nonhost trees (Hobson et al., 1993) or the presence of inhibitory nonhost, e.g., green leaf, volatiles (Zhang and Schlyter, 2004) in the same forest suggests that host

location by this beetle species must rely on multiple factors, such as the relative and absolute release rates of host and nonhost compounds, timing of response elicitation, and other life history aspects. Indeed, Pureswaran et al. (2004a, b) presented extensive evidence for several beetle/host combinations supporting the hypothesis that scolytid beetles utilize quantitative, as well as qualitative, differences in host odor blends in the process of host selection. Such a strategy would optimize host selection and minimize the risks associated with response to nonhost cues in the process of host location. In China, *D. valens* is faced with many new pine hosts that provide a continuous corridor for its spread from its region of introduction in northeastern China across Eurasia to Europe (Critchfield and Little, 1966). For example, *D. valens* colonizes *P. sylvestris* planted in North America (Wood 1982b); thus, it could be predicted that *D. valens* will infest naturally occurring *P. sylvestris* throughout Eurasia. Effective semiochemical lures are of increasing importance as this introduced pest expands its range in the Palearctic. If, as predicted, *D. valens* becomes more of a primary tree killer as a result of the effects of global warming, such methods will also become more important in its native range in the Nearctic.

Acknowledgement This study was supported by the USDA Forest Service, Forest Health Technology Enterprise Team (Morgantown, WV, USA), the National Natural Science Foundation of China (Project 30471403 and 30525009), the College of Natural Resources, University of California, Berkeley, and the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Universidad Autonóma Chapingo (Texcoco, Mexico), and the Chinese Academy of Sciences, Institute of Zoology (Beijing, China). We thank C.R. Rudolph (USDA Forest Service, Placerville, CA, USA), K. Bischel (USDA Forest Service, Berkeley, CA, USA), R. Acciavatti (USDA Forest Service, Morgantown, WV, USA), R. Murphy and M. McMahon (Department of Entomology, University of Wisconsin, Madison, WI, USA), S. Dahir (Wisconsin Department of Natural Resources, Madison, WI, USA) and D. Koenig (Carnegie Museum of Natural History, Pittsburgh, PA) for laboratory and field assistance.

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J Chem Ecol (2007) 33:147–156
DOI 10.1007/s10886-006-9208-7

Pentacyclic Triterpenes with Selective Bioactivity from *Sebastiania adenophora* Leaves, Euphorbiaceae

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Received: 2 February 2006 / Revised: 9 May 2006 / Accepted: 27 September 2006 /
Published online: 6 December 2006
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Abstract Six known pentacyclic triterpenes possessing oleanane, lupane, or taraxerane-type skeletons were isolated from the leaves of *Sebastiania adenophora* (Euphorbiaceae) and are reported for the first time in this species. These compounds include 3-*epi*-β-amyrin, β-amyrinone, 3-*epi*-lupeol, lupenone, taraxerol, and taraxerone. Structures were elucidated by comparison with literature data. The bioactivities of these compounds were tested on the root growth of *Amaranthus hypochondriacus*, amaranth (Amaranthaceae), *Lycopersicon esculentum*, tomato (Solanaceae), and *Echinochloa crus-galli*, barnyard grass (Poaceae). All six triterpenes were selectively bioactive. An important stimulatory effect was observed on amaranth root growth (23% to 56%) for almost all tested triterpenes (250 μg/ml). These triterpenes significantly inhibited the root growth of barnyard grass (28% to 78%) and tomato (23% to 49%). Aqueous leachate and organic extracts of *S. adenophora* leaves significantly inhibited the root growth of all test species. The possible ecological role of the allelochemicals isolated is discussed.

Keywords Allelopathy · Allelochemicals · *Sebastiania adenophora* · Euphorbiaceae · Pentacyclic triterpenes · Oleanane · Lupane · Taraxerane · 3-*epi*-β-amyrin · β-amyrinone · 3-*epi*-lupeol · Lupenone · Taraxerol · Taraxerone · *Amaranthus hypochondriacus* · *Echinochloa crus-galli* · *Lycopersicon esculentum*

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Introduction

Sebastiania adenophora Pax & K. Hoffm (Euphorbiaceae), commonly known as "zak chechén," "k'aan chunup," or "chechén blanco" is a tree up to 8–15 m high from the Peninsula of Yucatan, Mexico, Guatemala, and Belize (Martínez, 1994; Flores and Espejel-Carvajal, 1994). This tree is the main component of the *S. adenophora–Plumeria obtusa* var. *cericifolia–Agave angustifolia* forest community in Quintana Roo State. This community covers approximately 90% of the tropical forest area of this Mexican state (Sánchez-Sánchez and Islebe, 2002).

Sebastiania comprises over 101 species; 71 of them have been reported in the Peninsula of Yucatan, Mexico (Sousa and Cabrera, 1983). The empirical medicinal use of *S. adenophora* in this area is related to the use of the highly toxic latex of this tree. Two to three drops of it are used as a cathartic; a higher amount can cause death. In addition, *S. adenophora*'s wood is considered as rare and is appreciated in floors and furniture.

Phytochemical and pharmacological studies of *Sebastiania* species have yielded several phenolic compounds (Penna et al., 2001; Godoy et al., 1991; Calixto et al., 1990), steroids, and triterpenoids (Khera et al., 2003; Branco and Pizzolatti, 2002; Gaertner et al., 1999; Yunes et al., 1990). Some of these compounds have biological activity such as antimicrobial (Khera et al., 2003; Penna et al., 2001; Haraguchi et al., 1999; Godoy et al., 1991), antiviral (Kott et al., 1999), analgesic (Luzzi et al., 2000; Gaertner et al., 1999), or antispasmodic (Yunes et al., 1990; Calixto et al., 1990).

In a previous study (Romero-Romero et al., 2002), we reported the allelochemical effect of an aqueous leachate (1%) from the leaves of *S. adenophora* on the root growth and cytoplasmic protein synthesis patterns of maize, bean, squash, and tomato. The aqueous leachate exhibited a selective effect on root growth of the test species. Tomato roots were inhibited (70%) and squash roots were stimulated (49%). In addition, cytoplasmic protein pattern of all test plants was altered by *S. adenophora* aqueous leachate.

To continue with our studies on the allelochemical potential of *S. adenophora*, we isolated and identified the main secondary metabolites from leaves and searched for the compounds responsible for the differential effect on the growth of some plants.

Methods and Materials

Plant Material Leaves of *S. adenophora* were collected in Quintana Roo, Mexico, in February of 2004. Leaves were air-dried in the shade. A voucher specimen was deposited at the Herbarium of the University of Yucatan, Mexico.

Test Seeds Phytotoxicity of the aqueous leachate, organic extracts, and pure compounds from *S. adenophora* leaves were tested on the root growth of two dicotyledonous species: *Amaranthus hypochondriacus* L. (Amaranthaceae), amaranth, and *Lycopersicon esculentum* Mill. (Solanaceae) var. Pomodoro, tomato; and one monocotyledonous plant, *Echinochloa crus-galli* L. (Beauv.) (Poaceae), barnyard grass. We chose these three species to obtain rapid and more homogeneous results in bioassays. Amaranth seeds were purchased from a local market in Tulyehualco, Mexico, D.F. Barnyard grass seeds from Valley Seed Service, Fresno, California, USA and tomato seeds from Semillas Berentsen, Celaya, Guanajuato, Mexico.

Bioassays with Aqueous Leachate Two grams of *S. adenophora* dry leaves were soaked for 3 hr in 100 ml of distilled water. The leachate was filtered through Whatman paper (no. 4)

and then a sterile Millipore membrane (0.45 μm). The osmotic potential was measured with a freezing-point osmometer (Osmette A. Precision System), and the values ranged from 20 to 26 mosm/l=−0.045 to −0.059 MPa.

Aqueous leachate (2%, w/v) was mixed (1:1 v/v) with agar (2%) to obtain a 1% test solution. Pure agar (1%) was used as a negative control, and agar with 100 μg/ml of 2,4-D as a positive control. Seedling root growth bioassays for the aqueous leachate were carried out according to procedures published previously (Anaya et al., 2003). Bioassays were set up in 6-cm Petri dishes. Ten seeds of each test plant were sown directly on the agar of each Petri dish following a completely random design with 4 replicates per treatment. Petri dishes were kept in darkness at 27°C. Root lengths were measured 24 hr after treatment for amaranth, 48 hr for barnyard grass, and 72 hr for tomato. Data were analyzed by ANOVA and Tukey's tests (Mead et al., 2002).

Bioassays with Organic Extracts and Pure Compounds Bioassays with organic extracts (100 μg/ml) and pure compounds (250 μg/ml) were set up using the same conditions as for aqueous leachate, but with filter paper as a substrate. The filter paper (Whatman 42) was impregnated with 1.5 ml of the solutions or 2,4-D (250 μg/ml) as a positive control. After complete evaporation of solvents, filter papers were moistened with 1.5 ml of distilled water. Filter paper with only distilled water was used as negative control. Test seeds and time of harvesting for these bioassays were the same. IC_{50} (50% inhibitory concentration) values for 3-*epi*-β-amyrin (**1**), taraxerol (**5**), and the mixture of the six triterpenes (**E2** fraction), were calculated by probit analysis based on root growth bioassays where treatments were evaluated at 125, 250, and 500 μg/ml.

Extraction and Isolation of Allelochemicals in Leaves Plant material (2.0 kg) was sequentially extracted with hexane and CH_2Cl_2–MeOH at room temperature for 9 d. Evaporation to dryness under reduced pressure of the two extracts afforded a dark green residue.

The CH_2Cl_2–MeOH extract (40.1 g) was fractioned by column chromatography on silica gel (60, 70–230 mesh, Merck) using *n*-hexane–CH_2Cl_2–MeOH mixtures of increasing polarity, yielding 180 fractions (400 ml) that were reduced to 13 fractions (**A-M**) after comparison by thin layer chromatography (TLC) (pre-coated silica gel 60 F254 plates, Merck). Fraction **E** was selected for further fractionation because it had the highest activity in root growth bioassays with the dicotyledonous species amaranth and tomato, and the monocotyledonous species barnyard grass. From this primary fraction **E** (eluted with 30% *n*-hexane and 70% CH_2Cl_2), taraxerol (14-taraxeren-3-ol) [**5**] (4.2 mg) spontaneously crystallized (Sakurai et al., 1987; Ogihara et al., 1987). The mother liquors from this bioactive fraction were purified by silica gel column chromatography eluted with *n*-hexane, CH_2Cl_2, and MeOH in increasing polarity. The resulting fractions were combined according to TLC monitoring to give eight fractions (**E1**–**E8**). Bioactivity on plant growth was found in fraction **E2**. From this fraction (eluted with 60% *n*-hexane and 40% CH_2Cl_2), a mixture of the pentacyclic triterpenes **1**–**6** spontaneously crystallized. The mixture was resolved by preparative TLC and was eluted with CH_2Cl_2–MeOH (99.5:05) to give band I and II. Both bands were purified by successive preparative TLC (impregnated with a 10% $AgNO_3$ solution) eluted with CH_2Cl_2–MeOH (99.5:05). Band I yielded, 3-*epi*-β-amyrin (3α-hydroxyolean-12-ene) [**1**] (12.1 mg) [Tamai et al., 1989; Reyes et al., 1983] and 3-*epi*-lupeol (3α-hydroxylup-20(29)-ene) [**3**] (8.7 mg) [Carvalho and Seita, 1995; Waterman and Ampofo, 1985]. Band II yielded β-amyrinone (3-oxoolean-12-ene) [**2**] (7.6 mg) [Yan et al., 1989; Kikuchi et al., 1977], lupenone (3-oxolup-20(29)-ene) [**4**] (6.1 mg) [Carpenter et al.,

1980; Appleton and Enzell, 1971], and taraxerone (14-taraxeren-3-one) [**6**] (6.8 mg) [Parveen et al., 1988; Sakurai et al., 1987]. From the inactive fractions **H** and **L**, spontaneously crystallized β-sitosterol (45.3 mg) and β-D-glucositosterol (177 mg), respectively.

In contrast, the hexane extract (37 g) was subjected to silica gel column chromatography eluting with a gradient of *n*-hexane–CH_2Cl_2–MeOH. One hundred sixty fractions (200 ml each) were collected and pooled on the basis of their TLC profiles to yield 14 major fractions (**I**–**XIV**). Root growth bioassay showed one active pool (**VIII**). From fraction **VIII**, eluted with 30% *n*-hexane and 70% CH_2Cl_2, 26.0 mg of taraxerol (**5**) spontaneously crystallized and was recrystallized from a CH_2Cl_2–MeOH solution. The mother liquors from bioactive fraction **VIII** were also subjected to column chromatography on silica gel using *n*-hexane–CH_2Cl_2–MeOH mixtures of increasing polarity. This column yielded 125 fractions (50 ml each) that were reduced to 10 fractions (**VIII–1**–**VIII–10**) after comparison of their Rf's by TLC. According to the root growth bioassay, bioactivity was concentrated in secondary fraction **VIII–6**. From this fraction following TLC and elution with CH_2Cl_2–MeOH (99.5:05) 3-*epi*-β-amyrin (**1**) (8.2 mg) and taraxerol (**5**) (3.2 mg) were isolated.

General Experimental Procedures Melting points were determined on a Fisher–Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks on a Perkin-Elmer 599-B spectrophotometer. UV spectra were obtained on a Shimadzu 160 UV spectrometer in MeOH solution. Optical rotations were taken on a JASCO DIP 360 polarimeter. NMR spectra was recorded on a Bruker DMX500, in $CDCl_3$, either at 500 (^{1}H) or 125 (^{13}C) MHz, using tetramethylsilane (TMS) as an internal standard. EI mass spectra were performed using a Hewlet-Packard 5890 mass spectrometer.

Results and Discussion

Effects of Aqueous Leachate and Organic Extracts on Plant Root Growth The bioactivity of the aqueous leachate and organic extracts of the dry leaves of *S. adenophora* were determined (100 μg/ml) on the root growth of *A. hypochondriacus* (Amaranthaceae), amaranth; *L. esculentum* (Solanaceae), tomato; and *E. crus-galli*, (Poaceae) barnyard grass using 2,4-D (2,4-dichlorophenoxyacetic acid) as a positive control (Fig. 1).

The aqueous leachate of *S. adenophora* dry leaves inhibited root growth of tomato (63%) and amaranth (25%), with no effect on barnyard grass. The hexane extract inhibited root growth of amaranth (40%) and barnyard grass (35%), with no effect on tomato. The CH_2Cl_2−MeOH (1:1) extract inhibited root growth of amaranth (49%), barnyard grass (39%), and tomato (42%). These results led to selection of the hexane and CH_2Cl_2–MeOH (1:1) extracts for biodirected fractionation.

Isolation and Characterization of Compounds from S. adenophora Leaves Bioactivity-guided fractionation of the hexane and CH_2Cl_2–MeOH (1:1) extracts led to the isolation of six known pentacyclic triterpenes: 3-*epi*-β-amyrin (**1**), β-amyrinone (**2**), 3-*epi*-lupeol (**3**), lupenone (**4**), taraxerol (**5**), and taraxerone (**6**) (Fig. 2). This is the first report of these compounds in leaves of *S. adenophora*. Triterpenes **2** to **6** are reported for the first time in the genus *Sebastiania*. The spectral properties of oleanane, lupane, and taraxerane-type triterpenes, including IR, ^{1}H NMR, and ^{13}C NMR data, were identical to those previously reported in the literature (Carvalho and Seita, 1995; Yan et al., 1989; Tamai et al., 1989;

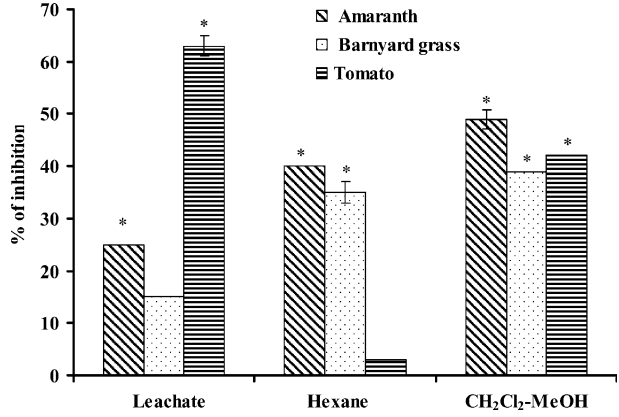


Fig. 1 Effect of aqueous leachate (1%), hexane, and CH_2Cl_2–MeOH extracts (100 μg/ml) of *Sebastiania adenophora* leaves on the root growth of amaranth, barnyard grass, and tomato. Vertical bars represent SD, $N=4$; $^*P<0.05$

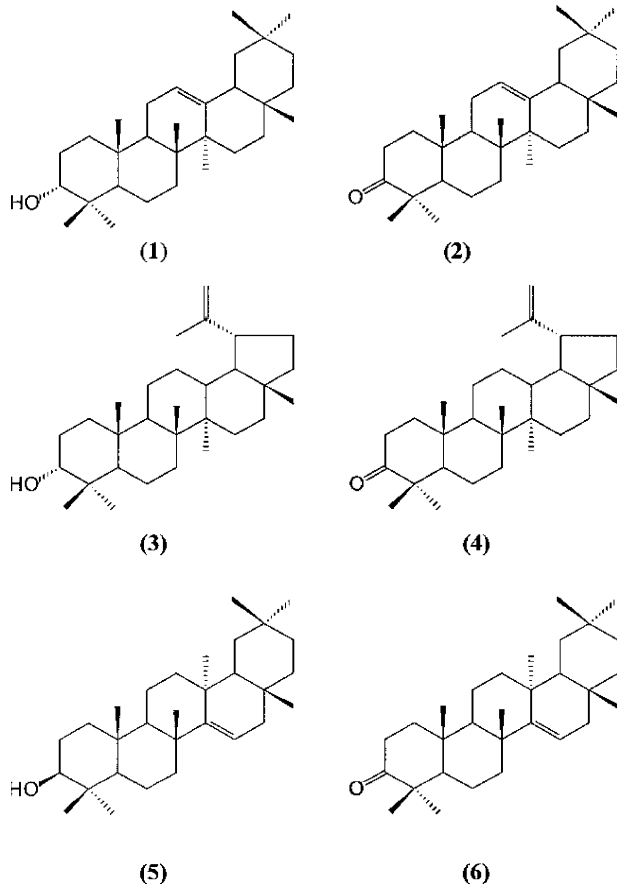


Fig. 2 Structures of pentacyclic triterpenes from *Sebastiania adenophora*: 3-*epi*-β-amyrin (**1**), β-amyrinone (**2**), 3-*epi*-lupeol (**3**), lupenone (**4**), taraxerol (**5**), and taraxerone (**6**)

Parveen et al., 1988; Sakurai et al., 1987; Ogihara et al., 1987; Waterman and Ampofo, 1985; Reyes et al., 1983; Carpenter et al., 1980; Kikuchi et al., 1977; Appleton and Enzell, 1971).

Langenheim (2003) mentioned that triterpenes dominate the nonvolatile composition of resins in some tropical families, particularly Burseraceae, Dipterocarpaceae, and Anacardiaceae. Resins of the Burseraceae typically have tetracyclic euphane/tirucallane, and pentacyclic lupane, ursane, and oleanane triterpene skeletal types. Resin is often listed as a component of latex in members of some families (e.g., Euphorbiaceae and Convolvulaceae). In the present study, we found that similar triterpenes were common in *S. adenophora* leaves, and they could be important components of its latex.

Bioactivity of Pentacyclic Triterpenes The bioactivity of triterpenes (250 μg/ml=5.9×10^{-4} M) was evaluated on root growth of amaranth, barnyard grass, and tomato (Fig. 3).

Six pentacyclic triterpenes stimulated amaranth root growth. The most effective compounds were 3-*epi*-β-amyrin (**1**) (55%), 3-*epi*-lupeol (**3**) (48%), and taraxerol (**5**) (39 %). These compounds contain a hydroxyl group at C-3. In contrast, pentacyclic triterpenes possessing oleanane and taraxerane-type skeleton showed phytotoxic effects on barnyard grass. Taraxerol (**5**) and taraxerone (**6**) were the most inhibitory on barnyard grass (77% and 42%, respectively). Root growth of tomato was inhibited by all triterpenes, particularly taraxerone (**6**) (50%) and taraxerol (**5**) (42%).

3-*epi*-β-amyrin (**1**), taraxerol (**5**), and the mixture of the six pentacyclic triterpenes (Fraction **E2**) were evaluated for their effect on root growth of amaranth, barnyard grass, and tomato at three different concentrations (125 μg/ml=2.9×10^{-4} M; 250 μg/ml=5.9×10^{-4} M; and 500 μg/ml=1.2×10^{-3} M). Figure 4 shows the effects of the different concentrations of these three treatments on the test plants. In general, 3-*epi*-β-amyrin (**1**), taraxerol (**5**), and the mixture of the six pentacyclic triterpenes (**1**–**6**) were stimulatory when tested on amaranth root growth. The effect increased with concentration. We estimate the 50% stimulatory concentration (SC_{50}) (required to stimulate 50% root growth) on amaranth for **1**, **5**, and **1**–**6** mixture (fraction **E2**) to be: 5.1×10^{-4} M, 7.6×10^{-4} M, and 372.4 μg/ml, respectively. The same three compounds inhibited root growth of barnyard grass and tomato in a concentration-dependent manner. Barnyard grass was inhibited most and taraxerol (**5**) produced the greatest inhibition on root growth of these two plants. The IC_{50} for barnyard grass was 4.38×10^{-4} M and for tomato, it was 6.23×10^{-4} M. IC_{50} values of

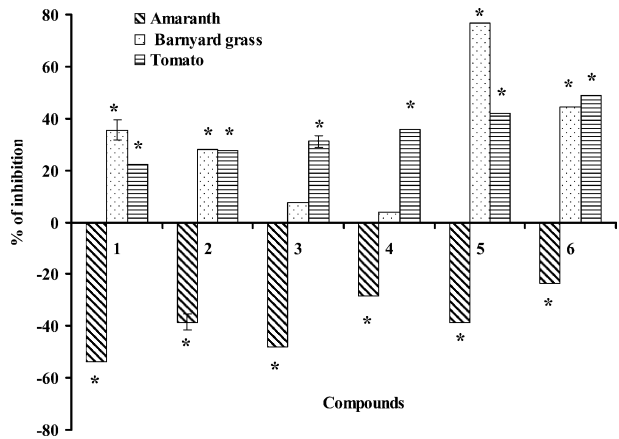


Fig. 3 Effect of the pentacyclic triterpenes (250 μg/ml or 5.9×10^{-4} M) isolated from *Sebastiania adenophora* leaves on the root growth of amaranth, barnyard grass, and tomato. A positive value represents inhibition; a negative value represents stimulation. Vertical bars represent SD, N=4; *P<0.05

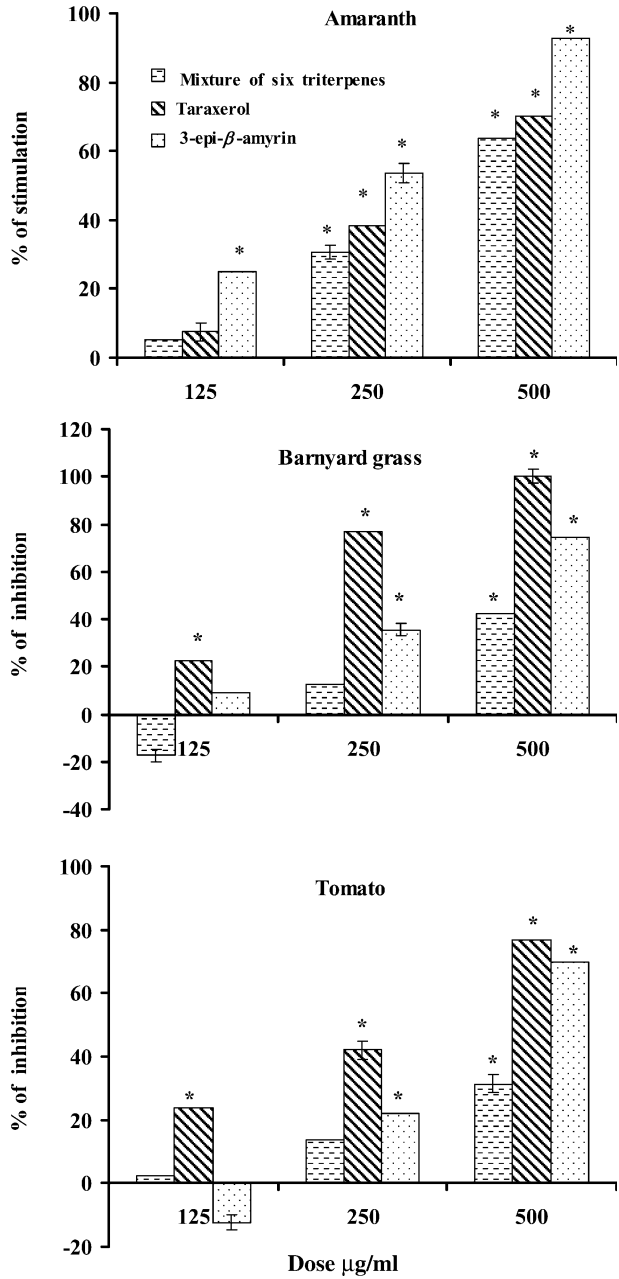


Fig. 4 Selective effects of 3-*epi*-β-amyrin (**1**), taraxerol (**5**), and the mixture of the six triterpenes at 3 different concentrations: [125 μg/ml (2.9×10^{-4} M); 250 μg/ml (5.9×10^{-4} M); 500 μg/ml (1.2×10^{-3} M)] on the root growth of amaranth (stimulatory effect); barnyard grass and tomato (inhibitory effects). Vertical bars represent SD, N=4; *P<0.05

the positive control 2,4-D were 1.88×10^{-4} M for barnyard grass and 2.3×10^{-4} M for tomato. The IC_{50} values for 3-*epi*-β-amyrin (**1**) were 7.30×10^{-4} M for barnyard grass and 8.72×10^{-4} M for tomato. The mixture of six triterpenes (fraction **E2**) had inhibited these two plants only at 500 μg/ml.

In previous studies, lupane and oleanane-type triterpenes exhibited clear selective bioactivity. These compounds could stimulate or inhibit germination or radical growth of

dicotyledonous and monocotyledonous species at concentrations ranging between 10^{-4} and 10^{-9} M. Root growth of the test plants responded differently, depending on the species and the substance tested. In general, triterpenes either stimulated germination of *Allium cepa* or inhibited root growth of this plant. In *Hordeum vulgare*, triterpenes inhibited germination of this monocotyledonous plant and stimulated its root growth (Macías et al., 1994, 1997, 1998a).

Macias et al. (1998b) found that some bioactive norsesquiterpenes isolated from *Helianthus annuus* exhibited clear selectivity on monocotyledon species with an average of inhibition of 45% on the germination of *A. cepa* and an average of stimulation of 50% on the root growth of *A. cepa* and *H. vulgare* in a range of concentrations of 10^{-5}–10^{-9} M.

The mechanism of allelopathic action of triterpenes is unknown. Duke and Oliva (2004) mentioned that the phytotoxic triterpenes holacanthone, chaparrinone, and glaucarubolone (quassinoids) inhibited some stages of mitosis. The former and the latter one also reduced chlorophyll accumulation. In addition, some saponins have effects on membrane properties because of their detergent-like activity.

Different plant species produce sesquiterpenoids and triterpenoids as defense compounds against pathogens (phytoalexins) and/or herbivores. Puglisi et al. (2004) found that aqueous and EtOAc extracts of the green alga *Penicillus capitatus* showed a strong inhibitory effect on the marine fungal pathogen *Lindra thallasiae*. Using *L. thallasiae* in bioassay-guided fractionation, they isolated two new cycloartane triterpene sulfate esters, capisterones A and B. Furthermore, Dutton et al. (2002) investigated the changes in apple leaf chemistry after infestation by leafminers and their effect on both host and habitat location of the generalist parasitoid *Pholetesor bicolor*. Chemical analysis of leaf solvent extracts from healthy and leafminer-damaged leaves revealed that herbivory increased the amount of the triterpene squalene ($C_{30}H_{50}$) that is an intermediate in the biosynthesis of sterols and triterpenes. Parasitoids performed a characteristic ovipositional probing more often on mine-damaged than on the healthy leaves.

Zinov'eva et al. (2001) mentioned the involvement of triterpenes in plant adaptation to stress induced by parasitic nematodes and pointed out that most studies were devoted to the biological activity of azadirachtin isolated from the neem tree (*Azadirachta indica* L., Meliaceae). This compound possesses insecticidal and antifeedant properties and inhibits the growth and molting of insects. Root exudates and lipid-containing extracts from neem seeds also suppress hatching and have nematocidal activity for some nematode larvae. Water extracts of leaves inhibit gall formation and fecundity of the nematode *Meloidogyne javanica* in tomato plants, but stimulate growth and productivity of these plants. The nortriterpenoid glycinoeclepin A derived from roots of common beans (*Phaseolus vulgaris* L., Fabaceae) and applied at a concentration of 6–10 mg/ml *in vitro* stimulates hatching of *Heterodera glycines* (Ichinohe) larvae.

Members of the cycloartane, lupane, ursane, oleanane, friedelane (especially quinone methides), dammarane, cucurbitacin, and limonoid triterpenoids, have anti-proliferative activity on various cancer cell lines (Setzer and Setzer, 2003).

In the present study, we described triterpenes **1–6** in *S. adenophora* leaves (**2–6** for the first time in *Sebastiania* genus) and confirm their selective bioactivity on the root growth of plants. Otherwise, the bioactivity of the mixture of allelochemicals in organic extracts and aqueous leachate of the leaves were phytotoxic, possibly due to a synergetic effect of the compounds present. Further study on the modes of action of these triterpenes will contribute to understanding the selective bioactivity on plants and on the physiological processes that result in inhibition or stimulation responses of plants.

Based on our results, we hypothesize that triterpenes and other allelochemicals that may be in the mixtures could play a multifunctional role in *S. adenophora*. These compounds act as alellopathics or as chemical defenses against herbivores, and may contribute to adaptation to the hostile environment of the dry tropical forest where this plant lives.

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J Chem Ecol (2007) 33:157–165
DOI 10.1007/s10886-006-9199-4

Interactions Between Host-plant Volatiles and the Sex Pheromones of the Bird Cherry-oat Aphid, *Rhopalosiphum padi* and the Damson-hop Aphid, *Phorodon humuli*

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Received: 15 May 2006 / Revised: 10 July 2006 / Accepted: 16 August 2006 /
Published online: 5 December 2006
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Abstract The bird cherry-oat aphid, *Rhopalosiphum padi* (L.), and the damson-hop aphid, *Phorodon humuli* (Schrank), migrate at the same time of year and colonize closely related *Prunus* spp. as primary hosts, but utilize (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol, respectively, as sex pheromones. Interactions between these sex pheromones and benzaldehyde and methyl salicylate, plant volatiles common to primary hosts of both species, were investigated to assess whether they confer reproductive isolation between these species. Female autumn migrants (gynoparae) and males of these two species were caught in the field with water traps baited with their respective sex pheromones. *Rhopalosiphum padi* gynoparae and males also responded positively to benzaldehyde. Release of either benzaldehyde or methyl salicylate with the conspecific sex pheromone increased catches of both species of aphid. However, releasing both plant volatiles with the sex pheromone of *R. padi* increased catches of gynoparae and males, but reduced those with the sex pheromone of *P. humuli*. These results support the hypothesis that specific plant volatiles synergize responses of autumn migrating aphids to their sex pheromone. Because these interactions are species-specific, they may be important in allowing males to discriminate between conspecific sexual females (oviparae) and those of other aphid species.

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Keywords Aphid · Aphididae · Sex pheromone · Nepetalactol · Benzaldehyde · Methyl salicylate · *Phorodon humuli* · *Rhopalosiphum padi* · Semiochemical

Introduction

The interaction between host-plant volatiles and sex pheromones of phytophagous insects, including aphids, is well established (Landolt and Phillips, 1997; Powell and Hardie, 2001). In many insect species, the response of males to a female-produced sex pheromone is directly influenced by host-plant volatiles. Host-plant volatiles may synergize the response to the sex pheromone, as has been shown for several species of Lepidoptera and Coleoptera with the simple addition of one or more green leaf volatiles (Light et al., 1993; Ruther et al., 2000, 2002; Reinecke et al., 2002a,b). Examples also exist where host-plant volatiles other than green leaf volatiles, or intact host plants, act synergistically with pheromones (Landolt and Phillips, 1997).

In contrast to the Lepidoptera and Coleoptera, for which many hundreds of species-specific sex pheromone components have been identified, of the 12 species of aphids for which sex pheromones are known, only three sex pheromone components have been identified (Hardie et al., 1999). There is also conflicting evidence as to whether male aphids can discriminate between the sex pheromone produced by conspecific sexually reproducing egg-laying females (oviparae) and those produced by oviparae of other species (Marsh, 1975; Eisenbach and Mittler, 1987; Steffan, 1990; Hardie et al., 1990, 1992).

The limited range of aphid sex pheromone components, and inconclusive evidence that aphids can discriminate between them, led Steffan (1987) and Guldemond (1990) to argue that the primary host plant may be important in promoting reproductive isolation. Subsequently, Powell and Hardie (2001) suggested that a more important role of host-plant volatiles may be to synergize the response of male aphids to sex pheromones. Gynoparae (female parthenogens that uniquely produce the sexually reproducing oviparae) of several species of aphid are known to respond to odors from primary host plants (Powell and Hardie, 2001) as do those and males also of the bird cherry-oat aphid, *Rhopalosiphum padi* (L.) and the damson-hop aphid, *Phorodon humuli* (Schrank). In olfactometer studies, gynoparae and males of *R. padi* responded positively to crushed buds of bird cherry, *Prunus padus* L., the primary host of this species (Pettersson, 1970). Subsequently, benzaldehyde was identified as an active component in the odor of bird cherry, eliciting positive behavioral responses on its own (Pettersson, 1970; Park et al., 2000). In contrast, in the field neither a steam distilled extract of bird cherry or benzaldehyde increased numbers of male *R. padi* caught in water traps (Hardie et al., 1994). However, when the steam distilled extract or benzaldehyde were released simultaneously with (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**I**), the sex pheromone of this species, significantly more males were trapped than with the pheromone alone, indicating a significant interaction. *Phorodon humuli* males responded positively to leaf and twig material from myrobalan, *Prunus cerasifera* Ehrhart, one of its primary hosts, as well as a bark extract from the same host in an olfactometer and in the field (Campbell et al., 1990). In a second study, steam distilled extracts of two other primary hosts, sloe (*Prunus spinosa* L.) and plum (*P. domestica* L.), also increased catches of gynoparae, but not males, in clear water traps (Lösel et al., 1996b). In addition, release of extracts from these hosts increased catches of gynoparae and males to the sex pheromone produced by conspecific oviparae, (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol (**III**) (Campbell et al., 1990; Lösel et al., 1996a,b).

These results suggest that primary host-plant volatiles elicit positive responses, both on their own and through synergistic interactions with sex pheromones, in autumn migrants of *R. padi* and *P. humuli*. However, gynoparae and males of *R. padi* and *P. humuli* migrate to closely related species of *Prunus*, which may provide similar olfactory cues. In addition, because the migrations occur at similar times of the year, there is no temporal separation. Thus, these two species of aphid respond to different sex pheromones but are presented with similar olfactory cues from their respective host plants. The responses of gynoparae and males of these species to their respective sex pheromones presented together with plant volatiles common to their primary hosts may provide insight into how aphids are able to make species-specific responses with such a limited range of sex pheromone components.

The present study investigated responses of gynoparae and males of *R. padi* and *P. humuli* to combinations of **I**, **III**, benzaldehyde, and methyl salicylate. The headspace profiles of *Prunus padus*, the primary host of *R. padi*, and *P. spinosa*, *P. domestica*, and *P. cerasifera*, primary hosts of *P. humuli*, are rich in volatile compounds including benzaldehyde and methyl salicylate (Pettersson, 1970; Pettersson et al., 1994; Pope, unpublished data). Benzaldehyde alone, as outlined above, is known to elicit positive responses in *R. padi* autumn migrants and to synergize responses to **I**. Methyl salicylate has been shown to evoke negative behavioral responses in spring migrants of both *R. padi* and *P. humuli*, which suggests some role for these specific compounds in primary host plant recognition (Campbell et al., 1993; Pettersson et al., 1994; Lösel et al., 1996a; Ninkovic et al., 2003).

Methods and Materials

Chemicals (4a*S*,7*S*,7a*R*)-Nepetalactol (**I**) was prepared from an extract of the catmint *Nepeta cataria* (L.) (see Dawson et al., 1987). A 7:3 mixture of the 1*S*- and 1*R*-diastereoisomers of (4a*R*,7*S*,7a*S*)-nepetalactol (**III**) was prepared from *N. racemosa* (syn. *mussinii*) (see Campbell et al., 1990, 2003). Benzaldehyde (**B**) and methyl salicylate (**MS**) were purchased from Sigma-Aldrich (Poole, UK) (purity 99% and 98%, respectively).

Field Experiments Randomized block factorial experiments were conducted in plantations of dwarf hops growing on 2.4-m-high trellises at East Malling Research (EMR) in 1996, 2000, and 2001. The experimental design consisted of two, four, and two blocks in 1996, 2000, and 2001, respectively. Water traps were made from transparent plastic Petri dishes (14 cm diam×2 cm deep) as described by Hardie et al. (1997). Traps were filled with a dilute solution of Lipsol, a nonionic detergent. Pheromones (10 mg) in 50 µl Et_2O and **B** (50 mg) were placed into individual glass vials (08-CPV Chromacol), with a 1-mm hole drilled through the cap. The antioxidant butylatedhydroxytoluene (BHT, 5 mg), was added to vials containing 50 mg of **B**. **MS** (41 mg) was placed into closed polyethylene vials (WP/5, Fisons) with four 1-mm holes drilled through the cap. Control vials were added as necessary so that all traps presented an identical visual image. Vials were suspended 1 cm above the surface of the water. The traps were spaced 3 m apart and 60 cm above the ground and sited centrally in the 3-m-wide alleys between the rows of hops. Traps were emptied and their positions rerandomized daily. Trapped *P. humuli* and *R. padi* gynoparae and males were counted in the laboratory.

The release rates of **B** and **MS** were determined by suspending vials within a fume cupboard with an air flow of 0.6 m sec^{-1} at 18±2°C. Weight changes in vials were recorded over a 7-d period and the release rates were calculated (mean±SE) as 1.03±0.02 mg day^{-1} for **B**, and 0.81±0.16 mg day^{-1} for **MS**.

The experiments were conducted from October 8 to 25, 1996, from October 14 to 30, 2000, and from September 22 to October 26, 2001. A complete factorial design was used in 1996 with each chemical at two levels (present and absent). The treatments were (1) **III**, (2) **B**, (3) **MS**, (4) **III**+**B**, (5) **III**+**MS**, (6) **B**+**MS**, (7) **III**+**B**+**MS**, and (8) control. Incomplete factorial designs were used in 2000 and 2001; all treatments that combined both sex pheromones were omitted. Therefore, the treatments in both years were (1) **I**, (2) **III**, (3) **B**, (4) **MS**, (5) **I**+**B**, (6) **I**+**MS**, (7) **III**+**B**, (8) **III**+**MS**, (9) **B**+**MS**, (10) **I**+**B**+**MS**, (11) **III**+**B**+**MS**, and (12) control.

Data Analysis Data were analyzed by analysis of variance (ANOVA). To stabilize variances, daily counts were transformed to square roots ($n^{0.5}$) and summed for the whole sampling period. A two-way classification of treatments within blocks was used for first-step analyses, with differences between means compared against the least significant difference (LSD) only if the overall treatment terms were significant. The data were then reanalyzed with the variance partitioned into factorial contrasts. Separate terms for block-to-block variance, main effects of each chemical individually, two- and three-chemical interactions, and residual variance for the incomplete factorial designs in 2000 and 2001 were accumulated into analysis of variance from regressions using GenStat 5 (Payne, 2000).

Results

A total of 2120 *P. humuli* gynoparae and 9764 males were caught in the 1996 experiment (Table 1). *P. humuli* gynoparae and males were more numerous in traps baited with **III** than in traps without pheromone. Furthermore, traps baited with **III** together with either **B** or **MS** caught significantly more gynoparae and males (1.9–2.1 and 2.5–2.7 times as many respectively) than did traps baited with **III** alone. The factorial ANOVA of these data showed that *P. humuli* gynoparae and males responded significantly to **III** (gynoparae, $F_{1,7}$= 71.57, P<0.001; males, $F_{1,7}$=162.93, P<0.001). The three-way interactions between **III**, **B**, and **MS** were also significant for both morphs (gynoparae, $F_{1,7}$=5.73, P<0.05; males, $F_{1,7}$=11.27, P<0.05). Both morphs responded positively to the plant volatiles, but only in the presence of **III** (**III**+**B** vs. **III**, gynoparae, t=2.95, P<0.05, males, t=3.69, P<0.01; **III**+**MS** vs. **III**, gynoparae, t= 3.11, P<0.05, males, t=3.98, P<0.01), but the response was negated when both plant volatiles were presented with **III** (**III**+**B**+**MS** vs. **III** gynoparae, t=1.33, males, t=0.82).

Table 1 Total numbers of *Phorodon humuli* gynoparae and males caught in water traps releasing benzaldehyde, methyl salicylate, and (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol (**III**) in 1996

Treatment	*P. humuli*	
	Gynoparae	Males
Control	110d	201c
Nepetalactol (**III**)	252bc	1213b
Benzaldehyde (**B**)	122cd	160c
Methyl salicylate (**MS**)	96d	177c
III+**B**	488a	3039a
III+**MS**	534a	3278a
B+**MS**	127cd	154c
III+**B**+**MS**	391ab	1542b

Numbers in a column followed by the same letter are not significantly different at P<0.05 based on two-way ANOVA of $n^{0.5}$ transformed counts.

Few gynoparae of *R. padi* and *P. humuli* were caught in the year 2000 experiment (29 and 1, respectively), which was conducted after the peak migration period for that morph. Totals of 441 *R. padi* and 368 *P. humuli* males were caught (Table 2). Males of *R. padi* responded significantly to their own pheromone (**I**) irrespective of the presence of the two plant volatiles, and more males responded to **I** released with **B**±**MS** (1.6–2.3 times as many) than to **I** alone. In contrast, catches of males in traps releasing **III** were no different from controls. The factorial ANOVA confirmed that *R. padi* males responded to **I** compared with either no pheromone (t=7.48, P<0.001) or **III** (t=6.24, P<0.001), as did gynoparae (**I** vs. control, t=2.16, P<0.05; **I** vs. **III**, t=2.27, P<0.05; **III** vs. control, t=0.11; data not shown). *Rhopalosiphum padi* males also responded to **B** (F=9.45, P<0.05) but not to **MS**. Although the two- and three-way interactions between pheromones, **B**, and **MS** were not significant at the 5% probability level, the two-way interaction between pheromones and **B** were close for both morphs (males, F=2.90, P=0.069; gynoparae F=2.63, P=0.087). Although these results must be treated with caution owing to the borderline significance of the F ratios, almost twice as many males responded to **B** when released with **I** compared with **I** alone (t=3.32, P<0.01) and a similar proportion to **B** alone compared with control (t=2.04, P<0.05). Gynoparae also responded to **B** when released together with **I**, as compared with **I** alone (t=2.58, P<0.05), but not otherwise.

The results for *P. humuli* males in 2000 were similar to those in 1996. They responded to **III**±**B** and **III**±**MS**, but the release of both plant volatiles with **III** halved the response. Males did not respond to **I**. The factorial ANOVA of these data confirmed that male *P. humuli* responded to **III** (**III** vs. control, t=7.25, P<0.001; **III** vs. **I**, t=7.22, P<0.001). Although the three-way interaction was not significant, an examination of the means showed that release of plant volatiles together with **III** reduced responses of males (**III** vs. **III**+**B**+**MS**, t=3.09, P<0.01).

Totals of 629 *R. padi* gynoparae and 606 males together with 104 *P. humuli* gynoparae and 597 males were caught in the 2001 experiment (Table 3). Owing to a shortage of pheromone, only two blocks of the experiment were deployed, and the distribution of *P. humuli* was skewed positionally with 94% of males and 80% of gynoparae trapped in one experimental block. This positional disparity increased the error variance and contributed to large differences in numbers trapped between treatments being not statistically significant. No such disparity in distribution was observed with *R. padi*. In comparison with **I** alone, 1.6–4.7 times as many gynoparae and 20–45 times as many males responded to **I** released with plant volatiles. The factorial ANOVA of these data confirmed that *R. padi* gynoparae

Table 2 Total numbers of *Phorodon humuli* and *Rhopalosiphum padi* males caught in water traps releasing benzaldehyde, methyl salicylate, and (4a*S*,7*S*,7a*R*)-nepetalactol (**I**)) or (1*RS*,4a*R*,7-*S*,7a*S*)-nepetalactol (**III**) in 2000

Treatment	*R. padi*	*P. humuli*
Control	8ef	1c
Nepetalactol (**I**)	46cd	2c
Nepetalactol (**III**)	19def	121a
Benzaldehyde (**B**)	15def	8bc
Methyl Salicylate (**MS**)	3f	1c
I+**B**	72ab	4c
I+**MS**	59bc	2c
III+**B**	38cde	122a
III+**MS**	19def	148a
B+**MS**	21cde	5c
I+**B**+**MS**	107a	2c
III+**B**+**MS**	10ef	58b

Numbers in a column followed by the same letter are not significantly different at P<0.05 based on two-way ANOVA of $n^{0.5}$ transformed counts.

Table 3 Total numbers of *Phorodon humuli* and *Rhopalosiphum padi* gynoparae and males caught in water traps releasing benzaldehyde, methyl salicylate, and (4a*S*,7*S*,7a*R*)-nepetalactol (**I**) or (1*RS*,4a*R*,7*S*,7a*S*)-nepetalactol (**III**) in 2001

Treatment	*R. padi*		*P. humuli*	
	Gynoparae	Males	Gynoparae	Males
Control	5d	4c	5ab	0b
Nepetalactol (**I**)	37cd	5c	4ab	1b
Nepetalactol (**III**)	8d	6c	15ab	153ab
Benzaldehyde (**B**)	59bcd	17c	7ab	4b
Methyl Salicylate (**MS**)	9d	5c	2b	2b
I+**B**	130ab	102b	5ab	2b
I+**MS**	60abc	224a	6ab	3b
III+**B**	78abcd	14c	12ab	340a
III+**MS**	18cd	2c	28a	83ab
B+**MS**	20cd	17c	4ab	1b
I+**B**+**MS**	172a	200ab	5ab	7b
III+**B**+**MS**	33bcd	10c	11ab	1b

Numbers in a column followed by the same letter are not significantly different at $P<0.05$ based on two-way ANOVA of $n^{0.5}$ transformed counts.

responded significantly to **I** compared either with no pheromone ($t=4.29$, $P<0.01$) or with **III** ($t=3.72$, $P<0.01$). *Rhopalosiphum padi* gynoparae responded to **B** as in previous trials ($F=10.54$, $P<0.01$), but not to **MS**. The factorial ANOVA showed that males of *R. padi* also responded to **I** compared either with no pheromone ($t=6.72$, $P<0.001$) or with **III** ($t=6.97$, $P<0.001$), but did not respond to **III**. In contrast to the 2000 experiments, *R. padi* males also responded to **MS** in 2001 ($F=9.17$, $P<0.05$), but not to **B**. The significant two-way interaction between pheromones and **MS** ($F=10.49$, $P<0.01$) showed that males responded to **MS** only when it was released with **I** (**I**+**MS** vs. **I**, $t=5.49$, $P<0.001$; **I**+**MS** vs. **MS**, $t=7.42$, $P<0.001$). The three-way interaction between pheromones and plant volatiles was not significant.

Gynoparae of *P. humuli* responded only to **III** in 2001 (Table 3). The factorial ANOVA of these data confirmed that gynoparae responded to **III** irrespective of the plant volatiles (**III** vs. no pheromone, $t=2.58$, $P<0.05$; **III** vs. **I**, $t=2.50$, $P<0.05$). Trap catches were unaffected by the presence of **I**. The two- and three-way interactions between pheromones, **B**, and **M** were not significant. Although the response of males to **III**+**B** was the only treatment combination greater than the control by two-way ANOVA, the factorial analysis showed that significantly more males were trapped in response to **III** than either to no pheromone ($t=2.81$, $P<0.05$) or to **I** ($t=2.67$, $P<0.05$). Gynoparae did not respond to either **B** or **MS**.

Discussion

Autumn migrants of both *R. padi* and *P. humuli* responded to the plant volatiles when the latter were released individually with their conspecific sex pheromone. The strength of these interactions differed between aphid species, with benzaldehyde inducing a stronger effect on *R. padi* than *P. humuli*. *Rhopalosiphum padi* males and gynoparae responded

significantly to the release of benzaldehyde in 2000 and 2001, and catches of both morphs were enhanced by the release of benzaldehyde with **I**. These results confirm those of Hardie et al. (1994), where extracts from bird cherry synergistically increased the number of males caught in the field in response to **I**, as did the release of benzaldehyde with **I**.

In contrast, *P. humuli* autumn migrants responded to benzaldehyde only when it was released with **III**, and then only in one of three years. Interestingly, in 1996, when responses to benzaldehyde were recorded, far more *P. humuli* were trapped in the 12.2 m Rothamsted Insect Survey suction trap at Wye (approximately 48 km from EMR) than in the two subsequent years of this study. The suction trap results showed that catches of *P. humuli* in 1996 were approximately 30 times greater than in 2000 or 2001.

Methyl salicylate elicited responses from *R. padi* and *P. humuli* only when released with the conspecific sex pheromone. In addition, responses were inconsistent, with methyl salicylate significantly increasing catches of *R. padi* males in 2001, and *P. humuli* gynoparae and males in 1996. Despite this, the responses of autumn migrants to methyl salicylate in the present study contrasts with the lack of response of spring migrants recorded previously (Campbell et al., 1993; Pettersson et al., 1994; Lösel et al., 1996a; Ninkovic et al., 2003). Therefore, our results offer moderate support for the suggested role of methyl salicylate as a cue allowing *R. padi* migrants to discriminate between primary and secondary hosts.

In contrast to the positive responses of the two aphid species to individual plant volatiles, responses of *P. humuli* males were reduced by the release of both plant volatiles with the conspecific sex pheromone. The same pattern was also evident for males in 2001. In addition, this response appeared to be species-specific because similar numbers of *R. padi* responded to the three-way combination and each two-way combination.

It is interesting to note that in spring, production of methyl salicylate by hop plants was increased from plants infested with *P. humuli* spring migrants (Campbell et al., 1993). Methyl salicylate acted as an antiaggregant, negating previously identified positive behavioral responses to (*E*)-2-hexenal and (−)-β-caryophyllene. Similarly, primary hosts of *P. humuli* also produced more methyl salicylate when infested with gynoparae (Wadhams, unpublished data.). Therefore, methyl salicylate may have a similar antiaggregation role in the response of *P. humuli* autumn migrants to **III** as does, for example, verbenone as an antiaggregant for the spruce bark beetle, *Ips typographus* (L.) (Zhang, 2003; Zhang and Schlyter, 2003).

The effect on catches of *P. humuli* males of releasing the plant volatiles with **III** depended upon whether benzaldehyde and methyl salicylate were released individually or together. Both of these plant volatiles are produced by primary hosts of *P. humuli*, and extracts of these hosts enhanced catches of gynoparae and males to **III** (Campbell et al., 1990; Lösel et al., 1996a,b). Therefore, the importance of other factors such as the ratio of compounds must be considered. Indeed, there is some evidence to suggest that aphids are able to discriminate between different ratios of **I** and (4a*S*,7*S*,7a*R*)-nepetalactone (Marsh, 1975; Eisenbach and Mittler, 1987; Steffan, 1990; Hardie et al., 1990, 1992). Aphids also discriminate between ratios of their common alarm pheromone, (*E*)-β-farnesene (EBF), and specific plant volatiles (Dawson et al., 1984). Dawson et al. (1984) noted that although hop plants also produce EBF, they are readily colonized by aphids, and that it was the corelease of (−)-β-caryophyllene by hop plants that allowed several aphid species, including *P. humuli*, to discriminate the source of EBF.

Rhopalosiphum padi and *P. humuli* are, to date, the only aphid species for which host-plant volatiles have been found to increase responses to sex pheromones (Campbell et

al., 1990; Hardie et al., 1994; Lösel et al., 1996a,b). The host-plant extracts used in those studies would have contained many of the same volatiles, given that the hosts are closely related species of *Prunus*, and likely included both methyl salicylate and benzaldehyde. The results presented here indicate that both benzaldehyde and methyl salicylate can affect the responses of *R. padi* and *P. humuli* autumn migrants to their respective sex pheromones, but show also that these responses are species-specific. Our results also provide some support for assertions that host plant volatiles and their interactions with aphid sex pheromones may be important in promoting reproductive isolation (Steffan, 1987; Guldemond, 1990; Powell and Hardie, 2001).

Acknowledgement T.W.P. was funded by the East Malling Trust for Horticultural Research and C.A. M.C. by the Department for Environment, Food and Rural Affairs. J.H. was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom. Rothamsted receives grant-aided support from the BBSRC.

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J Chem Ecol (2007) 33:167–181
DOI 10.1007/s10886-006-9185-x

Juvenile Hormone III Influences Task-Specific Cuticular Hydrocarbon Profile Changes in the Ant *Myrmicaria eumenoides*

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Received: 26 January 2006 / Revised: 07 July 2006 / Accepted: 05 August 2006 /
Published online: 5 December 2006
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Abstract We investigated the influence of juvenile hormones (JH) on the composition of cuticular hydrocarbons (CHCs) and the division of labor in colonies of the African ant *Myrmicaria eumenoides*. CHCs have long been implicated in nestmate recognition in social insect colonies. In *M. eumenoides*, the CHC profiles also vary with the task performed from brood-tender-type to forager type. The endocrine factors regulating the task allocation as well as the intracolonial recognition cues are not well understood, but JHs are prime candidates. Only JH III was identified in the hemolymph of *M. eumenoides* workers. Foragers had significantly higher JH III titers than brood tenders. The application of exogenous JH III and a JH analogue (methoprene) to *M. eumenoides* workers did not result in an observable acceleration of task change in our study. However, longevity of the focus workers, and thus the observational period, was reduced by the applications. Changes from a brood-tender-type to a forager-type CHC profile were accelerated by the application of JH III and methoprene, resulting in brood-tending workers that displayed forager-type CHC profiles. We present the first data supporting that recognition cues of an eusocial Hymenopteran are influenced by JH III, which could thus play a major role in the regulation of the dynamic nature of social insect colonies. JH III is connected to at least two key processes: the acceleration of CHC changes and the more long-term modulation of task shifting. Moreover, this indicates that changes in CHC recognition cues do not trigger task allocation in social insect colonies.

Keywords Cuticular hydrocarbons · Division of labor · Juvenile hormone · Social insects · Task

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Introduction

Ants (Formicidae) are ecologically among the most dominant organisms in the world (Hölldobler and Wilson, 1990). They are also highly social animals, and an important feature of social organization are the caste systems (Hölldobler and Wilson, 1990). Two basic castes are found in ants: reproductive females and males (fertile caste), and nonreproductive female workers (sterile caste; Wilson, 1971).

In some ant species, the sterile caste can be further subdivided into morphologically disparate subcastes, e.g., in *Pheidole dentata* (Johnston and Wilson, 1985) and *Atta cephalotes* (Wilson, 1983). Each subcaste is responsible for one or more particular tasks, resulting in polyethism (division of labor). However, caste polyethism is far less prevalent in ants than temporal polyethism (Hölldobler and Wilson, 1990), where the adult workers change tasks as they grow older. Usually, tasks inside the nest (e.g., brood tending) are executed early in life, whereas out-of-nest tasks (e.g., foraging) are performed later in life (Wilson, 1971). In the ant species *Pogonomyrmex barbatus*, workers can distinguish between nestmates engaged in tasks inside and outside the nest (Greene and Gordon, 2003). The nestmate recognition cues have been suggested to come from the cuticle (reviewed by Bradshaw and Howse, 1984).

The insect cuticle is covered by a thin lipid layer consisting to a large extent of hydrocarbons, and a fundamental function of this is to prevent water loss (Hadley and Schultz, 1987; Hadley, 1994; Blomquist et al., 1998). However, an additional major function of these cuticular hydrocarbons (CHCs) is to serve as recognition cues (reviewed by Howard and Blomquist, 2005). CHCs are involved in nestmate and species recognition in social insects (Jutsum et al., 1979; Nowbahari et al., 1990; Dahbi and Lenoir, 1998; Lahav et al., 1999; Wagner et al., 2000; Kaib et al., 2004). Direct experimental evidence supports the involvement of CHCs in nestmate recognition (Lahav et al., 1999; Thomas et al., 1999; Wagner et al., 2000). Furthermore, other studies have demonstrated that CHC profiles of workers within a colony are not uniform and may vary temporally (Nielsen et al., 1999; Cuvillier-Hot et al., 2001), with diet (Liang and Silverman, 2000), and with task (Kaib et al., 2000; Greene and Gordon, 2003), thus linking temporal polyethism and CHC recognition cues.

Both the development of fertile and sterile castes and the temporal division of labor in social insects are thought to be regulated by endocrine factors. Juvenile hormones (JH) play an important role in the reproductive division of labor in Hymenoptera (*Bombus terrestris*, Bloch et al., 2000; Bortolotti et al., 2001; *Apis mellifera*: e.g., Rembold et al., 1992), and Isoptera (*Zootermopsis angusticollis*, Brent et al., 2005).

In the Hymenoptera, JH also appears to be involved in temporal polyethism, e.g., in *Apis mellifera* (e.g., Robinson, 1985; Huang and Robinson, 1995), *A. cerana* (Huang et al., 2001), and *Polistes canandensis* (Giray et al., 2005). Numerous studies document the correlation between hemolymph JH titers and task performance in *A. mellifera*: the JH titer is low in brood tenders and high in foragers (e.g., Fluri et al., 1982; Huang et al., 1994; Huang and Robinson, 1995). Additionally, JH has been implicated in the production of foragers in *A. mellifera* (Bloch et al., 2002) and polybiine wasps (O'Donnell and Jeanne, 1998). In contrast, Cameron and Robinson (1990) could not find such a correlation in bumblebees, a species that does not exhibit a strong age polymorphism.

Because there is a correlation between task and CHC profiles, this suggests that JH also may be implicated in the composition of CHCs. It already has been shown that one or more steps of pheromone biosynthesis in blattodean and coleopteran species is influenced by JH (reviewed by Tillman et al., 1999). Thus, the aim of this investigation was to determine if JH plays a role in the temporal division of labor in *Myrmicaria eumenoides* colonies. This

was carried out in a two-pronged approach. First, the JH hemolymph titers of workers in particular task categories were measured, and second, the CHC profile changes with task in nontreated and hormone-treated workers were recorded, along with their behavioral development. The amalgamation of these results sheds some light on how the compositions of CHCs and task allocation in *M. eumenoides* colonies are governed.

Methods and Materials

Focus Colony, Marking, and Behavioral Observations

The *M. eumenoides* (Formicidae; Myrmicinae) focus colony was collected in the Kwale District of Kenya in June 1995. In the laboratory, the colony was maintained under constant conditions (25°C, 70% RH, 12:12 L/D) as described by Kaib et al. (2000). Relative humidity and temperature were similar inside and outside the "nest". Dots of various colors of acetone-based nail varnish in quantities of 0.3–0.6 μl were applied to the thorax of newly eclosed workers (callows) to individually mark them. These were then observed for 5 min every 24 to 48 hr (focal sampling) during their lifetime. All observations took place during the light phase between 0900 and 1900 hr, with location, behavior, and task being noted. Three task categories (brood tenders, "reserves", and foragers) and a developmental category (callows) were defined and observed.

Chemical Analysis

The noninvasive solid-phase microextraction (SPME) (Arthur and Pawliszyn, 1990) was used to extract CHCs from live *M. eumenoides* workers. A fiber with a 7 μm nonpolar polydimethylolsiloxane (PDMS) coating (Supelco; Sigma-Aldrich Chemie, Munich, Germany), which has been used successfully in other CHC profile studies on ants (Monnin et al., 1998; Cuvillier-Hot et al., 2001; Liebig et al., 2000; Dietemann et al., 2003), was used. CHC profiles were obtained by rubbing the fiber on the fourth tergite of the gaster for 5 min.

Samples were analyzed with a Hewlett-Packard 5890 Series II gas chromatograph (GC) equipped with a DB1 capillary column and flame ionization detector (FID) as described by Kaib et al. (2000) with a temperature program of 130°C 1 min, then rising to 180°C at 8°C/min, and finally to 300°C at 2°C/min. To ensure correct peak allocation a standardized *n*-alkane mixture (ThetaKit®, AccuStandard, CT, USA) was sampled at regular intervals.

Peaks were quantified by using the HP ChemStation® software. Fifty-one CHC components integrated and quantified across all individuals were chosen for statistical analysis. Coeluting components were treated as one component. The structural identification of *M. eumenoides* CHCs was previously carried out by Kaib et al. (2000) by using gas chromatography-mass spectrometry (GC-MS) and dimethyl disulfide (DMDS) derivatization.

CHC profiles of 14 untreated workers were sampled continuously across the three task categories in order to observe the general pattern of CHC profile changes in the colony.

JH Titer Determination

Pooled hemolymph samples from the task categories brood tender, "reserve", and forager (15–25 workers per sample; not the SPME-sampled workers) were used for the identification and quantification of JH. Hemolymph was collected with calibrated glass capillaries (Lamag, VWR, Germany), content to the nearest 0.1 μl was visually estimated

and the capillary was emptied into a conical vial containing 100 μl MeOH (HPLC-grade; Sigma-Aldrich Chemie, Schnelldorf, Germany) isooctane (HPLC-grade; Fluka, Taufkirchen, Germany) (1:1, v/v). The solvent/hemolyph ratio was at least 10:1 in all samples. Hemolymph sample preparation was carried out according to the method of Westerlund and Hoffmann (2004). The sample was centrifuged (2 times at 9,500*g*, for 15 min) in order to precipitate hemolymph proteins. The extracts were then concentrated and analyzed using liquid chromatography-mass spectrometry (LC-MS) on a Shimadzu mass spectrometer (LCMS-2010A) coupled to an Eldex MicroPro HPLC (Westerlund and Hoffmann, 2004). Separation was carried out with a C_{18} reverse-phase column (150× 2.0 mm, ReproSil-Pur ODS-3, 5 μm; Dr. Maisch GmbH, Ammerbuch, Germany) H_2O/MeOH at a flow rate of 200 μl/min. Total run time was 15 min; 0–5 min isocratic at 30% MeOH, 5–10 min from 30% to 100% MeOH, 10–12 min from 100% back to 30% MeOH. The column temperature was kept constant at 37°C. The mass spectrometer was operated in electrospray (ESI) positive ion mode with a gas flow (N_2) of 3.76 L/min, a curved desolvation line (CDL) temperature of 250°C, and a heat block temperature of 200°C. The system was operated in Q-array scan mode. Peaks were integrated by using the "LC-MS Solution by Shimadzu" program, and quantified by using an external calibration curve based on known amounts of JH III across several orders of magnitude. The limit of detection was determined to be 6 pg.

Juvenile hormone detection and identification was carried out by matching of retention times with those of authentic standards, as well as the monitoring of the following diagnostic ions: *m/z* 289, 267 (JH III [M + Na^+]+ and [M + H]+ adducts), *m/z* 303, 281 (JH II [M + Na^+]+ adducts), and *m/z* 317, 295 (JH I [M + H]+ adducts). Only JH III was identified in our samples.

JH III and Methoprene Application

For topical applications, the JH analogue methoprene [isopropyl (2*E*,4*E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate] and racemic JH III [methyl-(2*E*,6*E*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate; Fluka, Steinheim, Germany] were dissolved in acetone (HPLC grade; Sigma-Aldrich, Taufkirchen, Germany). The applied dose of JH III was 50 pmol (0.13 pg), whereas that of methoprene was 1.77 nmol (0.55 μg). A CHC profile (to serve as reference profile) was taken from each focus worker prior to the first application. One μl of acetone (control), JH III, or methoprene solution was then applied twice a week to the head of the focus workers. These were kept individually in ventilated Petri dishes for 30 min after application of the solutions to ensure all the acetone had evaporated. The age of the workers at the first application was standardized to 4±1 d.

Data Analyses

Data were analyzed using the Statistica 5 (1997) package. Where the properties of the data sets allowed (Lilliefors Normality Test, and Levene's Homogeneity of Variances Test passed) Analyses of variance (ANOVAs) were carried out. Where necessary, *post-hoc* comparisons were then made using Tukey's HSD test. Where the assumptions of normal distribution and variance homogeneity were not met, data were subjected to the nonparametric Kruskal–Wallis *H* test, or a Kruskal–Wallis ANOVA. In this case, the *post-hoc* comparisons were carried out by using the Critical Difference Test for mean ranks: critical $\text{difference}_{0.05} = 2.394\sqrt{(N(N+1)/12 * (1/n_1 + 1/n_2))}$, where N is the total number of subjects in the experiment, and n_1 and n_2 are the number of subjects in the groups compared.

Calculations to estimate the contribution of individual peaks to the total variance between CHC profiles were carried out using the variance components module contained in the Statistica 5 (1997) package. A maximum likelihood approach was used to estimate the variance components. The statistical theory underlying maximum likelihood variance component estimation techniques is detailed by Searle et al. (1992).

The percentage contribution (relative concentration) of each component to the total CHC fraction was calculated to allow multivariate analyses. A similarity index (Nei distance 072, Nei, 1972; see also Nowbahari et al., 1990) for pairwise comparisons between each CHC profile was calculated and this was then subjected to a principal components analysis (NT-SYS 2.02). The first three principal components were taken as an estimate of CHC variation, and the number of dimensions responsible for the estimate of CHC profile variation was reduced from three to one by calculating the Euclidean distances between the relevant profiles (Statistica 5, 1997).

The first CHC profile after eclosion of each focus worker (equivalent to $x=0$, $y=0$, $z=0$) was taken as the reference point from which the Euclidean distances to other CHC profiles of this worker were calculated. Hence, the resulting one-dimensional CHC variation measurement ("chemical distance") represents vectors from zero. Low chemical distances stand for CHC profiles similar to the reference profile, and *vice versa* for high chemical distances.

Results

Temporal Polyethism and CHC Profile Variation

We identified three distinct task categories in the behavioral development of *M. eumenoides* workers. Workers carried out brood-tending duties early in life (up to 8 wk), and then went through an intermediate "reserve" phase (characterized by a high degree of inactivity) before eventually changing to foraging duties (Fig. 1). This behavioral transition was continuous and took place in a similar fashion in all focus workers, thereby confirming the presence of temporal polyethism in colonies of *M. eumenoides*. However, the time required for the transition from interior to exterior work varied from worker to worker; this parallels the findings of Otto (1962) on *Formica polyctena* workers. The CHC profiles of the workers were shown to gradually change in and parallel to this task transition.

The CHC profiles consist of a complex mixture of substances representing a series of *n*-alkanes, alkenes, and alkadienes with a chain length of 21–36 C atoms. The mean of CHC profiles collected during repetitive sampling of each focus worker in a particular task category was calculated. We found a large intraindividual variation in CHC profiles of individual workers performing different tasks (Fig. 1). To assess this variation, the mean "chemical distances" of the profiles were calculated by using the callows group centroid as reference, and a global significance was found when the three task groups were compared (repeated-measures ANOVA, $N=42$, $F=41.69$, $P<0.001$). The CHC profiles of the brood tenders (mean ± SD: 0.155±0.043, $N=14$) changed continuously and in a synchronous fashion as a function of time towards a "reserve"-type CHC profile (0.341± 0.069, $N=14$). This change is significant (Tukey's HSD test, $P<0.001$), but the change from a "reserve"-type CHC profile to a forager-type CHC profile (0.357±0.086, $N=14$) was not (Tukey's HSD test, $P=0.805$). This means that the interindividual CHC profile variation within the brood-tending task category was lower than the intraindividual variation between each of the brood tenders and their profiles during the "reserve" or foraging phases, respectively.

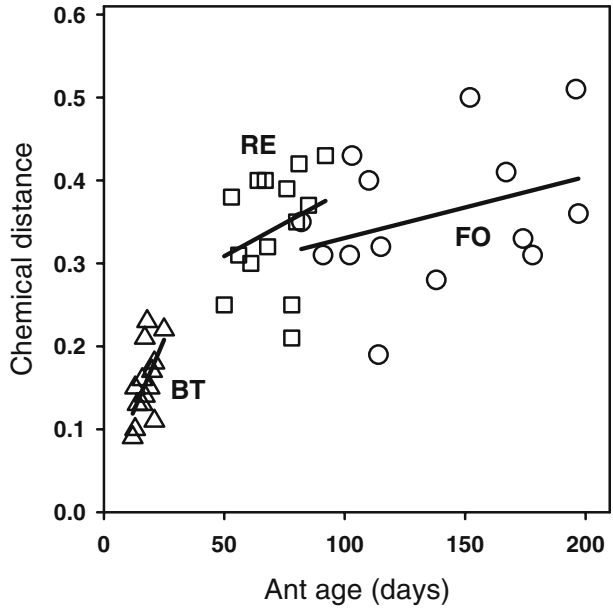


Fig. 1 Variation of CHC profiles in 14 *M. eumenoides* workers as a function of age and task. The brood tender (BT, ▵), "reserve" (RE, □), and forager (FO, ○) symbols represent the mean number of days post eclosion plotted against the mean chemical distances of all CHC profile samples taken from each individual in that task category (r^2_{BT}= 0.34, r^2_{RE}=0.08, r^2_{FO}=0.12). Chemical distance is the Euclidean distance to the callows group centroid; black lines are linear regressions for the CHC profiles of the three different task categories

All task groups were shown to have the same 51 CHCs—this means that the CHC profile change from brood tender to forager was only quantitative. Using variance components analysis, the difference between the CHC profiles of brood tenders and foragers was shown to be more than 98% due to only 10 components: four *n*-alkanes, two alkenes, and four alkadienes (Table 1, series I). In terms of relative abundance, these 10 components were classed as major- and mid-sized components.

Juvenile Hormone Identification and Hemolymph Titers

Only JH III was identified in the hemolymph samples of all workers. A global significance was found when the JH III titers (expressed in pg/μl hemolymph) of the brood tenders, "reserves", and foragers were compared (ANOVA, N=30, F=6.45, P=0.005) (Fig. 2). Foragers (N=10, 32.6 ±10.9) had a significantly higher JH III hemolymph titer (Turkey's HSD test, P=0.003) than brood tenders (N=10, 15.4±7.5). JH III titers of "reserves" (N= 10, 23.2±13.0) were intermediate. Differences were not significant when brood tenders and "reserves", or foragers and "reserves" were compared (Turkey's HSD test, P=0.24 and P= 0.14, respectively). This demonstrated the existence of a task-related difference in JH III hemolymph titers among *M. eumenoides* workers. The JH III titers of hormone- and hormone analogue treated workers were not measured because the workers would have had to be killed in order for us to carry out the tests. Thus, they would no longer have been available for the continuous SPME sampling of CHCs during their entire life span.

Effects of the Application of JH III and Methoprene on Behavioral Development

All application treatments including the control had detrimental effects on the longevity of the test subjects, reducing their life expectancy to only 4–6 wk after the first application. None of the treated individuals were observed to forage at any point. Thus, the mean

Table 1 Comparison of CHCS that together contribute over 98% to the variance between brood-tender-type and forager-type CHC profiles

Compound	Series I[a]			Series II[b]		
	BT	FO	Variance	BT	JH-appl. BT	Variance
n-Heptacosane	3.77±0.76	13.39±3.44	48.22	4.58±2.12	8.93±4.05	15.77
7,21+7,23+7,25-Untriacontadiene	21.66±1.63	15.86±2.35	17.37	26.51±2.31	21.06±2.62	26.29
n-Pentacosane	11.98±1.67	7.80±2.59	8.80	9.46±3.99	3.94±1.24	26.63
7,21+7,23+7,25-Nonacosadiene	4.77±1.21	1.60±0.24	5.23	4.77±1.05	2.25±0.74	5.67
7,21+7,23-Tetratriacontadiene	6.00±1.21	9.22±0.91	5.35	4.77±1.29	7.59±1.78	6.87
7-Hentriacontene	2.63±0.44	5.29±0.77	3.68	3.22±0.39	4.06±0.86	0.58
Dotriacontadiene	23.84±1.66	27.59±3.17	6.86	25.54±4.65	28.84±4.20	6.95
n-Nonacosane	1.87±0.41	3.40±0.75	1.21	1.52±0.44	3.47±0.97	3.38
n-Untriacontane	1.20±0.44	2.59±0.64	1.00	0.67±0.21	2.90±0.94	4.48
4+5-Tritriacontene	1.09±0.19	2.30±0.33	0.77	0.79±0.23	2.14±0.34	1.66
Total			98.49			98.29

Values for BT and FO are given as mean ± SD of the relative abundance, and variance values state the % contribution to the total variance.

[a] Series I represents a comparison of CHC profiles of untreated brood tenders and foragers.

[b] Series II represents a comparison of CHC profiles of untreated brood tenders and brood tenders applied with JH III leading to forager-type CHC profiles (JH-appl. BT).

number of days post first application that a worker started to show "reserve" behavior was recorded (control, 31.5±7.5; 50 pmol JH III, 28.1±7.8; methoprene, 28.4±9.5) as a representative measure of behavioral development. When the hormone-treated groups were compared to the control group, no difference regarding the start of the "reserve" phase was found (ANOVA; N=22, F=0.724, P=0.33).

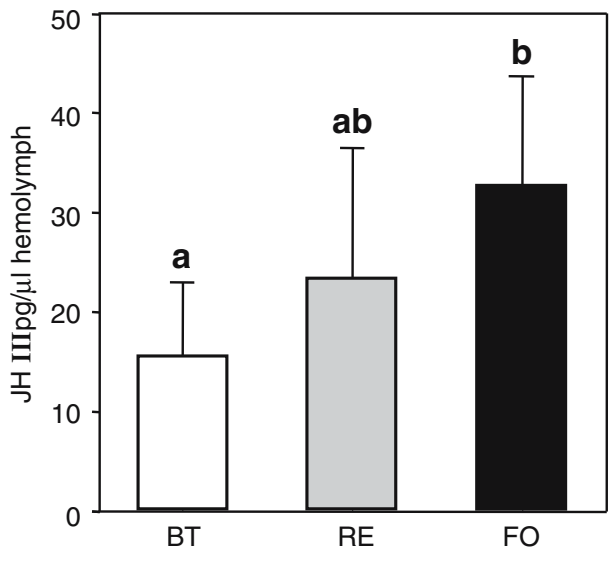


Fig. 2 The JH III hemolymph titers (mean ± SD) of brood tenders (BT), "reserves" (RE), and foragers (FO). Each category is represented by 10 pooled samples. Different letters denote significant differences at the P<0.05 level

Effects of the Application of JH III and Methoprene on CHC profiles

Effects of the application of JH III and methoprene on CHC profiles were documented in terms of days post first application, although none of the treated workers changed from brood tending to foraging. The five different time brackets (Fig. 3) were analyzed, and in the first no significant difference in comparison to the control group was found (Kruskal–Wallis $ANOVA_{1-10}$; $N=19$, $H=2.16$, $P=0.34$). However, in the other four time brackets significant differences were found (Kruskal–Wallis $ANOVA_{11-20}$; $N=21$, $H=8.62$, $P=0.013$, Kruskal–Wallis $ANOVA_{21-30}$; $N=21$, $H=9.87$, $P=0.007$, Kruskal–Wallis $ANOVA_{31-40}$; $N=18$, $H=8.08$, $P=0.017$, Kruskal–Wallis $ANOVA_{41-60}$; $N=14$, $H=9.00$, $P=0.011$).

The application of 50 pmol JH III resulted in increased chemical distances (measurement of magnitude of CHC profile change) in all time brackets, but significant differences between JH III treated workers and controls were only found from days 21 to 30 (Critical $Difference_{0.05}$, $P<0.05$), and from days 41 to 60 (Critical $Difference_{0.05}$, $P<0.05$). For days 31–40, the value obtained was below the critical difference value (Critical $Difference_{0.05}$, $P>0.05$) and was thus not significant, but due to the highly conservative nature of the *post-hoc* test used this result is nevertheless viewed as presenting a clear trend. The application of methoprene also resulted in increased chemical distances in all time brackets, and these were significant in comparison to the control group from day 11 post application onwards (Critical $Difference_{0.05}$, $P<0.05$ in all cases). In sum, these results suggest that the CHC composition in *M. eumenoides* workers is influenced by JH III.

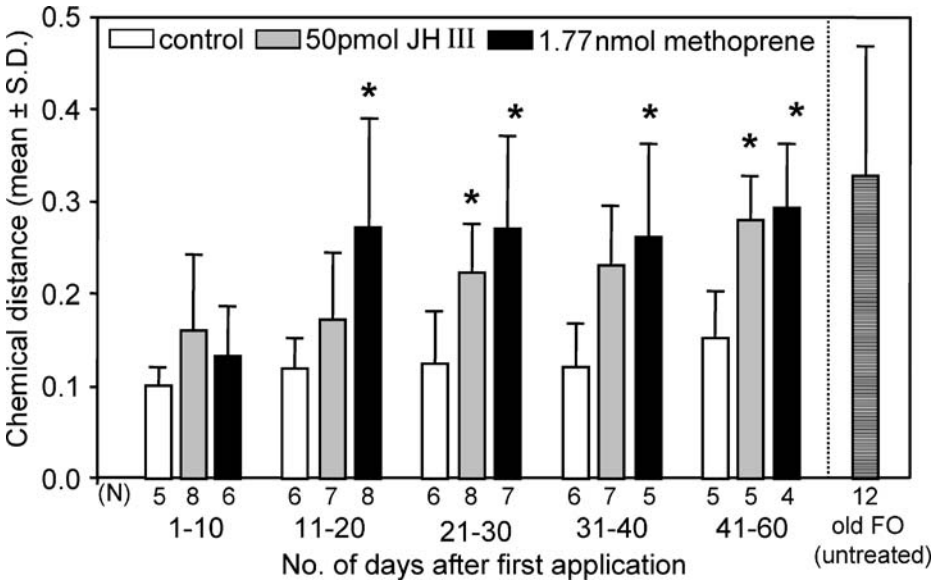


Fig. 3 Effects of application of 50 pmol JH III, 1.77 nmol methoprene, or acetone controls, on the CHC profiles of individual workers as a function of time. Chemical distance is the Euclidean distance to the first CHC profile taken from a focus worker prior to the first application. Thus the mean chemical distance of CHC samples in each time bracket was first calculated per individual, then for each application group. The number of individuals (N) used in the analysis is denoted in each time bracket for each application category. Asterisks indicate differences at the $P<0.05$ level between that specific application group and the acetone control group in the same time bracket. The "old foragers" (old FO) group was included for comparison

Analysis of Application-Induced CHC Profile Changes

Variance components analysis showed that CHC profiles of the control group (performing brood-tending duties) can be differentiated from CHC profiles of the treated brood tenders altered through JH III and methoprene application on the basis of only 10 components (Table 1, series II). These 10 compounds made up more than 98% of the total variation between the CHC profiles. Moreover, these were the same compounds that were shown to summarize the differences between brood-tender-type and forager-type CHC profiles observed during the course of the normal behavioral development in untreated workers. This suggests that the application of JH III, as well as its analogue methoprene, resulted in a precocious shift from brood-tender-type towards forager-type CHC profiles.

Discussion

We investigated the influence of JH III and its analogue methoprene on CHC profile changes and the division of labor in *M. eumenoides*. The endocrinological control of the division of labor in social insect colonies has already been the subject of several studies (Sasagawa et al., 1989; Huang et al., 2001; Schulz et al., 2002). However, to the best of our knowledge, this marks the first time that the endocrinological basis of CHC profile changes has been investigated specifically and parallel to age-related task changes in the same workers.

Our results showed that behavioral transitions took place during the life of all untreated focus workers. These carried out brood-tending duties early in life, and then went through the "reserve" phase before eventually changing to foraging duties. The CHC profiles of workers changed parallel to the task transitions. These results therefore suggest a strong correlation of CHC profiles and task allocation in the ant *M. eumenoides*, in agreement with the findings of Kaib et al. (2000). Our data also showed that foragers have higher JH III hemolymph titers than brood tenders, and thus a strong correlation between CHC profile changes and JH III titers exists in *M. eumenoides.* When we topically applied JH III and methoprene, we found that the CHC profiles of workers changed towards a forager-type CHC profile after the applications. However, we did not observe that task change was affected when focus workers were treated with JH III and methoprene.

Unfortunately, we found that the life span of workers was greatly reduced by the application of acetone (solvent) during the application experiments. The toxic effects of organic solvents at the cellular level are well known, but only poorly documented. However, it is generally agreed that the cell membrane is the primary target, and that changes in permeability lead to increased mortality (e.g., Stevens and Hofmeyr, 1993). In addition, the life span of workers was further reduced by high doses of JH; this type of toxic effect has been previously noted (e.g., Kuhr and Cleere, 1973; Meola et al., 2001; Orth et al., 2003). Thus, our applied concentrations represented a trade-off between worker longevity and the amounts of JH III that we were planning to apply (within the range of the hemolymph titers).

The type of behavioral transition observed in untreated *M. eumenoides* workers is in good agreement with the basic temporal polyethism pattern observed in eusocial Hymenopterans. This has been described as strikingly similar from species to species (Hölldobler and Wilson, 1990): tasks inside the nest are executed early in life, whereas out-of-nest tasks are performed later in life. This has been thoroughly investigated in the honeybee, *Apis mellifera* (Giray and Robinson, 1994; Giray et al., 1999), as well as the ants

P. barbatus (Wagner et al., 1998) and *Diacamma ceylonense* (Cuvillier-Hot et al., 2001). We also found that in *M. eumenoides* the time required for the transition from interior to exterior work (the "reserve" phase) varied from worker to worker (data not presented here), paralleling findings by Otto (1962) on *F. polyctena*.

The factors regulating temporal polyethism, i.e., task allocation, in social insect colonies have as yet not been determined, but increasing evidence indicates the involvement of JH (Robinson and Vargo, 1997; Hartfelder and Emlen, 2005). It is generally accepted that JH III is the only form of JH found in Hymenoptera (Gäde et al., 1997). Our results support this; we only found JH III in *M. eumenoides* workers, regardless of the task performed. In addition, several published studies showed that high JH III hemolymph titers are associated with foraging, whereas low JH III titers are associated with within-nest duties such as brood tending in, e.g., *B. terrestris* (Bloch et al., 2000), *Diacamma* spp. (Sommer et al., 1993), and *A. mellifera* (Robinson, 1987; Huang et al., 1991). Again, our results parallel these findings, because the JH III hemolymph titers of foragers were shown to be significantly higher than those of brood tenders. Studies on the honeybee *A. mellifera* showed that JH III and methoprene treatments promoted a precocious switch from activities in the nest to foraging (Robinson, 1985; Robinson et al., 1989; Sasagawa et al., 1989). In contrast, a study on *B. impatiens* and *B. bimaculatus* reported that application of 250 μg methoprene, and the injection of 250 μg JH I did not lead to precocious foraging (Cameron and Robinson, 1990), possibly because these two *Bombus* spp., unlike *A. mellifera* and most of the Formicidae, do not exhibit strong age-related polyethism.

We did not see a precocious task switch when we applied both JH III and its analogue methoprene to *M. eumenoides* workers, a species demonstrating a strong temporal polyethism. This discrepancy might be explained by the fact that the concentrations of JH III (0.13 pg/μl) and methoprene (0.55 μg/μl) applied in our study were between 2 and 4 orders of magnitude lower than those administered in the above study, and might have been too low to have any effect on task allocation. As stated earlier, higher concentrations of JH III would have markedly reduced the period of survival of the treated workers, and would have made the collection of CHCs from individual workers over longer periods impossible.

Alternatively, it has been suggested that JH III may only act as a "behavioral pacemaker" and is not necessary for the onset of foraging behavior *per se* (Robinson and Vargo, 1997; Sullivan et al., 2000). Such a "pacemaker" effect is likely to take place over a longer period, and we may not have been able to observe this due to the application-related premature deaths of the focus workers. In our study, JH III- and methoprene-treated workers survived for only 4–6 wk posteclosion, whereas nontreated workers started to forage roughly 18 wk after eclosion.

Besides playing a role in the temporal division of labor, our data provide evidence that JH III is also involved in regulating chemical communication in social insects. Signaling in the Hymenoptera appears to be primarily based upon CHCs (reviewed by Howard and Blomquist, 2005). Numerous studies have shown the involvement of CHCs in nestmate recognition, especially in the ants (Lahav et al., 1999; Thomas et al., 1999; Wagner et al., 2000; Lucas et al., 2005). Task-related CHC profiles are also thought to occur in *A. mellifera* (Francis et al., 1989), and several studies have documented the close correlation between hemolymph JH III titers and task in this species (Robinson et al., 1989; Huang and Robinson, 1995). This correlation gives an indication that CHC profiles in eusocial Hymenoptera workers are influenced by JH III. In our study, we also found that CHC profiles of *M. eumenoides* workers varied with the task performed (brood tending, foraging), and also strongly correlated with their hemolymph JH III titers. In addition, our study is the first to document the effects of topically applied JH III and methoprene on CHC

profile changes. Compellingly, we found that the application of these substances to *M. eumenoides* workers resulted in a general, precocious shift of the CHC profiles from brood tender type to forager type, resulting in brood-tending workers that displayed clear forager-type CHC profiles. These artificially induced CHC profile changes took place in a continuous fashion, altered the CHC profile within a short period, and intensified with increasing numbers of applications. Moreover, these changes were induced at concentrations roughly 1 order of magnitude below the physiological JH III titers of the workers, and several orders of magnitude below the concentrations applied or injected in comparative studies (regarding the effect of JH III on temporal polyethism).

In untreated *M. eumenoides* workers, the CHC profile changes took place in a continuous fashion from a brood-tender-type to a forager-type profile, and only quantitative involved changes. A quantitative shift of 10 saturated and unsaturated components (*n*-alkanes, alkenes, and alkadienes) within the whole CHC profile typified the change from a brood-tender-type to a forager-type CHC profile. The CHC profile changes induced by the application of JH III and methoprene involved the same 10 components documented in the CHC profile changes of untreated workers. The resulting CHC profiles were indistinguishable from the "normal" forager-type CHC profiles produced during the behavioral transitions that take place over much longer periods. This indicates that the same underlying biosynthetic pathways are affected when JH III is applied, and suggests the involvement of JH III in the CHC profile changes, and thus in signaling.

In contrast to the general hydrocarbon biosynthesis pathways in insects (reviewed by Howard and Blomquist, 2005), there is no information on how JH III could mould the CHC composition in social insects. From studies on pheromones of nonsocial insects, there are indications that JH III may act directly via a receptor-mediated gene induction for a key enzyme at some point along the biosynthesis pathway (Schal et al., 1994, 2003; Tillman et al., 1998; Tittiger et al., 2003). However, in contrast to these pheromones, which usually consist of only one or a few substances, the CHCs of social insects constitute a far more complex system of recognition cues. Thus, the fact that CHC profile changes in hormone-treated workers parallel the "normal" CHCs shift with task in nontreated workers (i.e., a change in the same 10 components) suggests that enzymes responsible for chain elongation, desaturation, and polydesaturation are affected by JH III. Thus JH III may exert its influence at several points along the biosynthetic pathway.

Another likely regulatory point is at the CHC transport level: high-density lipophorin (HDLp) has been hypothesized to play a key role in the transportation of CHC from the site (s) of synthesis to the cuticle (reviewed in Schal et al., 1998). Additionally, HDLp has a specific, high-affinity JH III binding site in Hymenoptera (Sevala et al., 1997). Because *M. eumenoides* individuals carrying out different tasks have altered relative proportions of the same CHCs, it is conceivable that JH III regulates the loading and/or unloading of CHCs into HDLp. Interestingly, it was recently demonstrated that the HDLp-bound hydrocarbons in the ant *Pachycondyla villosa* are quantitatively and qualitatively similar to the CHCs (Lucas et al., 2004).

Finally, our data also showed that "task signaling" takes place in colonies of *M. eumenoides*, paralleling findings from several other ant species (*Camponotus vagus*, Bonavita-Cougourdan et al., 1993; *P. barbatus*, Wagner et al., 1998, 2001; *Diacamma ceylonense*, Cuvillier-Hot et al., 2001). It appears that these task-specific CHC profiles allow workers to determine the tasks performed by nestmates during brief antennal encounters. Greene and Gordon (2003) demonstrated that workers perceive and respond to task-related CHC differences in the red harvester ant *P. barbatus*. In light of the results obtained in our study, this suggests a JH III-mediated, task-based recognition cue system in

M. eumenoides colonies. Such an endocrinologically controlled recognition system could allow each worker to assess the workforce involved in the various tasks on a small spatial scale (i.e., a subsample of the colony). This would allow individual workers to assess the colony requirements using CHCs as simple, localized cues, and to adjust their own behavioral development and signal composition accordingly. Tentative evidence is provided by Gordon and Mehdiabadi (1999), who demonstrated that rates of brief antennal contact (involving the perception of CHCs) influence task decisions in the ant *P. barbatus*. Altogether, this would result in a dynamic network that efficiently regulates a colony's behavior (Greene and Gordon, 2003) without any central or hierarchical control. Although this type of scenario may constitute the backbone of temporal polyethism in eusocial Hymenopterans, it is likely that other environmental and social cues also play a role in the "task decisions" made.

In summary, our investigation has shown that hemolymph JH III titer, task, and cuticular recognition cues in *M. eumenoides* workers are linked, but apparently two independent processes exist. First, short-term JH III application only leads to CHC profile changes, and acts at low JH III hemolymph concentrations. Second, a more long-term process that perhaps requires higher JH III concentrations modulates the task shift from brood tender to forager. Furthermore, these results also indicate that changing the task-specific CHC signals is not a prerequisite for temporal polyethism.

Acknowledgment We thank Antje Halwas for help and support during the investigations, and we are grateful to Konrad Fiedler for advice regarding statistical problems. This study was supported by the Deutsche Forschungsgemeinschaft as part of the Graduiertenkolleg 678 at the University of Bayreuth.

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J Chem Ecol (2007) 33:183–198
DOI 10.1007/s10886-006-9210-0

Seasonal Variation in Volatile Compound Profiles of Preen Gland Secretions of the Dark-eyed Junco (*Junco hyemalis*)

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Received: 26 May 2006 / Revised: 18 September 2006 / Accepted: 20 September 2006 /
Published online: 5 December 2006
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Abstract Quantitative stir bar sorptive extraction methodology, followed by gas chromatography-mass spectrometry (GC-MS) and element-specific atomic emission detection (AED) were utilized to analyze seasonal changes in volatile components of preen oil secretions in *Junco hyemalis*. Juncos were held in long days to simulate breeding conditions, or short days to simulate nonbreeding conditions. Linear alcohols (C_{10}–C_{18}) were the major volatile compounds found in preen oil, and in both sexes their levels were higher when birds were housed on long as opposed to short days. Methylketones were found at lower levels, but were enhanced in both sexes during long days. Levels of 2-tridecanone, 2-tetradecanone, and 2-pentadecanone were also greater on long days, but only in males. Among carboxylic acids (C_{12}, C_{14}, and C_{16}), linear but not branched acids showed some differences between the breeding and nonbreeding conditions, although the individual variation for acidic compounds was large. Qualitatively, more sulfur-containing compounds were found in males than females during the breeding season. Functionally, the large increase in linear alcohols in male and female preen oil during the breeding season may be an indication of altered lipid biosynthesis, which might signal reproductive readiness. Linear alcohols might also facilitate junco odor blending with plant volatiles in the habitat to distract mammalian predators. Some of the volatile compounds from preen oil, including linear alcohols, were also found on the wing feather surface, along with additional compounds that could have been of either metabolic or environmental origin.

Keywords Avian volatile compounds · Preen oil · *Junco hyemalis* · Stir bar sorptive extraction · Gas chromatography-mass spectrometry

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Introduction

In birds, the uropygial (also called "preen") gland produces lipids, much like those produced by mammalian sebaceous glands. These lipids are spread over the feathers as the bird uses its bill to preen. As an example, the junco first rubs its bill against the gland located on its rump, and then applies the secretions as it runs individual feathers between its upper and lower mandibles (Schrock, personal observation). Among other functions, the oils may protect the feathers from wear (Stettenheim, 1972), aid in waterproofing (Elder, 1954; Fabricius, 1959; Elowson, 1984), or protect against dermatophytes (Jacob et al., 1997) including lice (Moyer et al., 2003).

Traditionally, the composition of preen oil gland secretions has focused on nonvolatile compounds and has been investigated by hydrolyzing the waxes (mostly nonvolatile compounds) under basic conditions into acids, acid esters, and alcohols. Long-chain and branched acids and their esters were thus found to be the main components in hydrolyzed wax collected from the preen gland of the mute swan (*Cygnus olor*) (Odham, 1965) and also from waterfowl belonging to the avian family *Anatidae* (Odham, 1967). Even carbon-numbered long-chain alcohols were also recovered from hydrolyzed preen wax in a shorebird, the red knot (*Calidris canutus*) (Dekker et al., 2000).

Several investigations of the waxy composition of preen gland secretions have compared the nonbreeding and breeding seasons and reported shifts from monoester to diester waxy components in sandpipers (*Scolopacidae*) and in male and female Mallard ducks (*Anas platyrhynchos*) (Bohnet et al., 1991; Reneerkens et al., 2002). It was hypothesized for the sandpipers (*Scolopacidae*) that a shift from more volatile monoester preen waxes in winter to less volatile diester preen waxes in spring and summer may reduce the "odor" of the bird's feathers causing it to transfer less of this odor to the nest when incubating, and thus making the nest less noticeable to olfactorily sensitive mammalian nest predators (Reneerkens et al., 2002). Alternatively or in addition, the diester waxy preen oil during summer may alter the sandpiper's appearance, perhaps by influencing UV reflectance or other aspects of visual attractiveness, and thus play a role in mate choice (Reneerkens et al., 2002). Bohnet et al. (1991) suggested that compounds from the preen gland, when spread over the feathers, may also influence mate choice through a potential sex pheromone action. Preen oil may even have a role in advertising the allelic status of an individual's MHC genes to potential mates via a particular odor (Freeman-Gallant et al., 2003).

Recently, in studies with the great snipe (*Gallinago media*), which is a lekking bird species, a correlation between the MHC lineage and a mating success has been confirmed (Ekblom et al., 2004). The olfaction-mediated functions in birds, more than was previously appreciated, are plausible in light of new evidence for a stronger role for olfaction in passerines and other avian orders (see Roper, 1999 for a review).

Junco hyemalis is a classic species for the study of seasonality, as it was the first wild species shown to respond to changes in day length (summarized in Nolan et al., 2002). *J. hyemalis* is a socially monogamous, ground-nesting bird. Plasma testosterone levels have been reported to vary seasonally in both males and females (Schoech et al., 1998; Ketterson et al., 2005). Although the female builds the nest and does all the incubation, the male also provides parental care by delivering food to the young in the nest (Nolan et al., 2002).

To date, relatively few studies of any bird species have reported the composition of the volatile fraction of the preen oil. As with the contents of waxy compounds, the composition of volatile compounds reported in preen gland secretions and feathers also appears to differ greatly depending on the bird species. A "citrus odor" found on the feathers of the crested

auklet (*Aethia cristatella*) has been found to contain a group of aldehydes (hexanal, octanal, decanal, Z-4-decenal, and 12-carbon unsaturated aldehyde) that act as the major volatile chemosignals (Douglas et al., 2001). It was originally suggested that these aldehydes are capable of repelling invertebrate species, bacteria, and fungi, and consequently might signal an individual's health and vigor in the mate selection process.

A more recent study has shown that the crested auklet feather aldehydes are effective as mosquito repellants (Douglas et al., 2005). In addition, Hagelin et al. (2003) established that the "tangerine odor" in volatile secretions (octanal and *cis*-4-decenal) of the crested auklet feathers act as social odors to present to conspecifics. Partner-specific olfactory recognition was also observed between Antarctic prions (*Pachiptila desolata*) by Bonadonna and Nevitt (2004), but the specific compounds involved were not identified. Foul-smelling preen gland secretions (consisting of acids, aldehydes, ketones, amines, and dimethyl sulfide) in the green woodhoopoe (*Phoeniculus purpureus*) serve as a multifunctional tool, repelling predators and warding off feather-degrading bacteria (Burger et al., 2004).

In this paper, we report on the volatile and semivolatile chemical composition of the preen gland secretions of male and female dark-eyed juncos (*J. hyemalis*) during the breeding and nonbreeding seasons. Sorptive stir bar extraction methodology (Soini et al., 2005) with gas chromatography-mass spectrometry (GC-MS) was utilized in the investigation of the preen oil compositions. A novel *in situ* stir bar sampling method (Soini et al., 2006) was also applied to study volatile compounds on the surface of the wing feather.

Methods and Materials

Preen Oil Collection Sample secretions were collected from captive juncos in breeding and nonbreeding conditions. The juncos were originally captured near Pembroke, VA, USA and were held thereafter at the Kent Farm Bird Observatory in Bloomington, IN, USA. Their diet consisted of millet seed and a pureed mixture of dog chow, hard-boiled eggs, and carrots supplemented with vitamins and mealworms, and was available *ad libitum*. A total of 20 individuals were used, including 10 males and 10 females, and each individual was sampled first under short days and then under long days in a repeated measures design. Both short- and long-day conditions were maintained for 8 wk before the sample collection. The short-day birds (8L:16D) are referred to as "nonbreeding," whereas the long-day birds (16L:8D) are referred to as "breeding." In fact, neither group bred, but the long-day birds exhibited indicators of reproductive readiness, i.e., males developed large cloacal protuberances and females developed brood patches (defeathered areas on the ventral surface that transfer heat to developing embryos). Air temperature also differed by season: nonbreeding, short-day birds were held at −2–4°C, whereas in the breeding-season, long-day birds were held on at 18–20°C. Temperature conditions are typical temperatures juncos would experience in the wild population at Mountain Lake Biological Station in Virginia during the normal seasonal cycle.

Preen gland contents were collected by gently squeezing the area around the gland until a small amount of oil was discharged (Kolattukudy et al., 1987). The preen oil was then collected with a 100-μl microcapillary glass pipette that was weighed before and after sampling to determine how much was collected. The experimental procedures were approved by the Bloomington Institutional Animal Care and Use Committee of Indiana University. The samples (1.3–1.8 mg) were frozen at −20°C for the gas chromatography-

mass spectrometry (GC-MS) and gas chromatography-atomic emission detection (GC-AED) analyses.

Reagents and Materials All compound identifications were verified through authentic standards. The standard compounds and ammonium sulfate (99% +) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). The stir bars (Twister™, 10 mm, 0.5-mm film thickness, 24-μl polydimethylsiloxane, PDMS volume) used for the sorptive extraction were purchased from Gerstel GmbH (Mülheim an der Ruhr, Germany).

Preparation of Preen Oil Samples Volatile and semivolatile compounds were extracted from the preen oil samples by placing a 100-μl glass capillary containing the sample in a 20-ml capped glass vial with 2 ml of water (high-purity OmniSolv® water from EM Science, Gibbstown, NJ, USA), 100 mg of ammonium sulfate, and a Twister™ stir bar. As an internal standard, 8 ng of 7-tridecanone (Aldrich) was added in 5 μl volume of methanol to each vial. The stirring speed was 800+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnic, Oberschleissheim, Germany) for a 60-min extraction time. Before extraction, all glassware was washed with acetone and dried at 80°C. After extraction, the stir bars were rinsed with a small amount of distilled water, dried gently on the paper tissue, and placed in the TDSA autosampler tube (a product of Gerstel) for the GC or GC-MS analysis.

Collection of Volatile Compounds from Surface of the Wing Feather A rolling stir bar method (Soini et al., 2006) was applied in the collection of wing feather volatiles from the live bird wings. A wing feather of a single junco was sampled, rolling a stir bar gently over about 4 cm^2 surface area of its length. Sample-containing stir bars were subsequently stored in a refrigerator. The analyses were performed within 48 hr after collection. Stir bars were placed in the thermal desorption autosampler (see the description of equipment) without any further sample manipulation.

Analytical Instruments The GC equipment for quantitative analyses consisted of the Agilent 6890N gas chromatograph connected to a 5973i MSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE, USA) with the Thermal Desorption Autosampler and Cooled Injection System (TDSA-CIS 4 from Gerstel). Positive electron ionization (EI) mode at 70 eV was used with the scanning rate of 2.47 scans/sec over the mass range of 40–350 amu. A MSD transfer line temperature was set at 280°C. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The separation capillary was DB-5MS (20 m×0.25 mm, i.d., 0.25 μm film thickness) from Agilent (J&W Scientific, Folsom, CA, USA).

Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection assembly, CIS-4. TDSA operated in a splitless mode. Temperature program for desorption was 20°C (0.5 min), then 60°C/min to 250°C (3 min). Temperature of the transfer line was set at 280°C. CIS was cooled with liquid nitrogen to −80°C. After desorption and cryotrapping, CIS was heated at 12°C/sec to 280°C, with the hold time of 10 min. CIS inlet was operated in the solvent-vent mode, a vent pressure of 14 psi, a vent flow of 50 ml/min, and a purge flow of 50 ml/min. The temperature program in the GC operation was 50°C for 2 min, then increasing to 200°C at the rate of 3°C/min (hold time, 10 min). The carrier gas head pressure was 14 psi (flow rate, 1.1 ml/min at the constant flow mode).

Element-selective compound profiling was performed using a GC 6890 instrument equipped with an Atomic Emission Detector (AED, model G2350A) from Agilent Technologies (Wilmington, DE, USA) and a Thermal Desorption Autosampler-Cooled Injection System (TDSA-CIS-4 from Gerstel). The separation capillary was an HP-5MS (30 m× 0.25 mm, i.d., 0.25 μm film thickness) from Agilent. Samples were thermally desorbed in a TDSA automated system, followed by injection into the column with a cooled injection assembly under the same conditions as described above for the GC-MS analysis, except that the CIS was cooled with liquid nitrogen to −60°C. The temperature program in the GC operation was 50°C for 2 min, then increasing to 200°C at the rate of 3°C/min. The final temperature was held for 12 min. The carrier gas head pressure was 14 psi (flow rate, 1.2 ml/min). The GC unit was operated at the constant flow mode. The emission lines for carbon (193 nm), sulfur (181 nm), and nitrogen (174 nm) were monitored during the atomic plasma emission detection.

Quantitative Comparisons A total of 24 samples were quantitatively analyzed by GC-MS, six from each group (breeding and nonbreeding females and males). Four other samples from each of the 4 groups were qualitatively screened for the sulfur compounds by the GC-AED method. Of the approximately 100 compounds detected in chromatographic profiles of the preen oil by GC-MS, about 40 were tentatively identified. All major compounds were positively identified through comparison of their mass spectra and retention times to those of standard substances. Peak areas of the identified compounds were used to make quantitative comparisons between the long- and short-day groups of each sex. Peak areas were integrated either from the TIC (total ion current) profiles or from the postrun, selected ion current (SIC) profiles at *m/z* 55, *m/z* 58, and *m/z* 60. Peak areas of the internal standard were integrated from the *m/z* 113 profiles. Peak areas of the compounds of interest were normalized by dividing each peak area by that of the internal standard in corresponding runs. Reproducibility of the internal standard peak area was 13% (relative standard deviation, RSD, $N=12$).

Statistical Analyses Statistical comparisons of the normalized peak areas were calculated by using nonparametric, two-tailed Mann–Whitney matched pairs *U* tests for seasonal comparisons within sex and simple Mann–Whitney *U* tests for sex comparisons within a season. Statistically significant values of $P<0.05$ were accepted.

Results and Discussion

The volatile compound GC-MS profiles (collected under the scan mode over 40–350 amu) were complex when viewed as total ion current chromatograms (TICs). The profiles consisted of the major components (peak intensity 50–2,000 times over the detection limit), along with a large number of minor components (peak intensity 10–50 times over the detection limit). Because of this degree of complexity, not all compounds were fully resolved in the GC separation. Figure 1 illustrates a compound pattern that is representative of the "raw" volatile compound profiles. To facilitate more precise comparisons of the peak area integrations, we employed a postrun modification of the TIC profiles known as selected ion current (SIC) profiles, which allowed us to view the data "one ion" at a time.

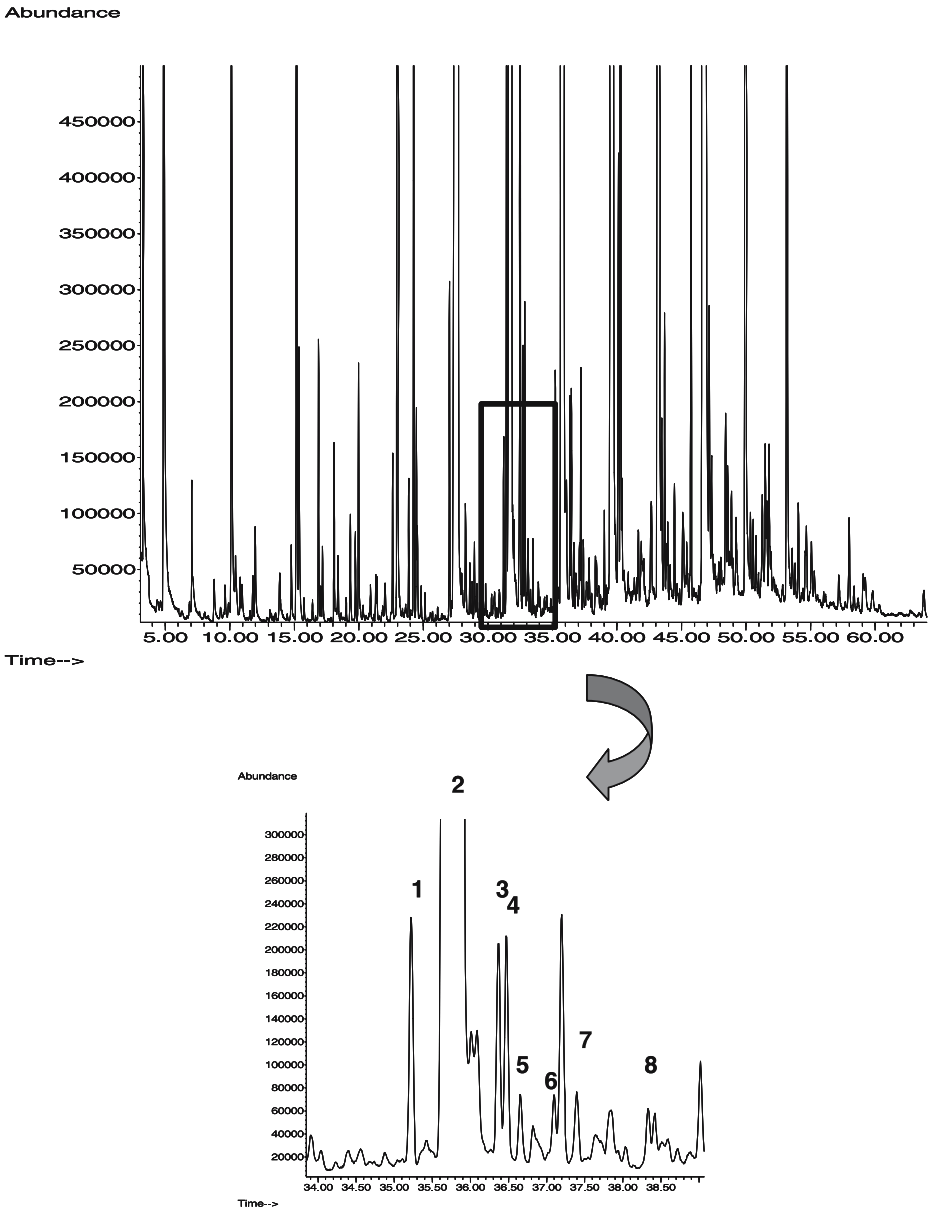


Fig. 1 A representative GC–MS TIC (total ion chromatogram) of the volatile compound profile extracted from preen oil. The enlargement of the framed area shows complexity of the profile leading to a partial co-elution of compounds. Numbers indicate the compounds 1: a methyl carboxylic acid, 2: 1-tridecanol, 3: 1-hexadecene, 4: 2-tetradecanone, 5: hexadecane; 6: 1-tetradecanol, 7: benzophenone, 8: a branched hydrocarbon

In addition to the TIC profiles, ions *m/z* 55, 58, and 60 were chosen to monitor peak areas of alcohols and aldehydes (*m/z* 55), a series of methylketones (*m/z* 58), and fatty acids (*m/z* 60). In Fig. 2, we demonstrate typical simplified profiles for *m/z* 55 and *m/z* 60. Six females and 6 males were investigated during both breeding and nonbreeding conditions

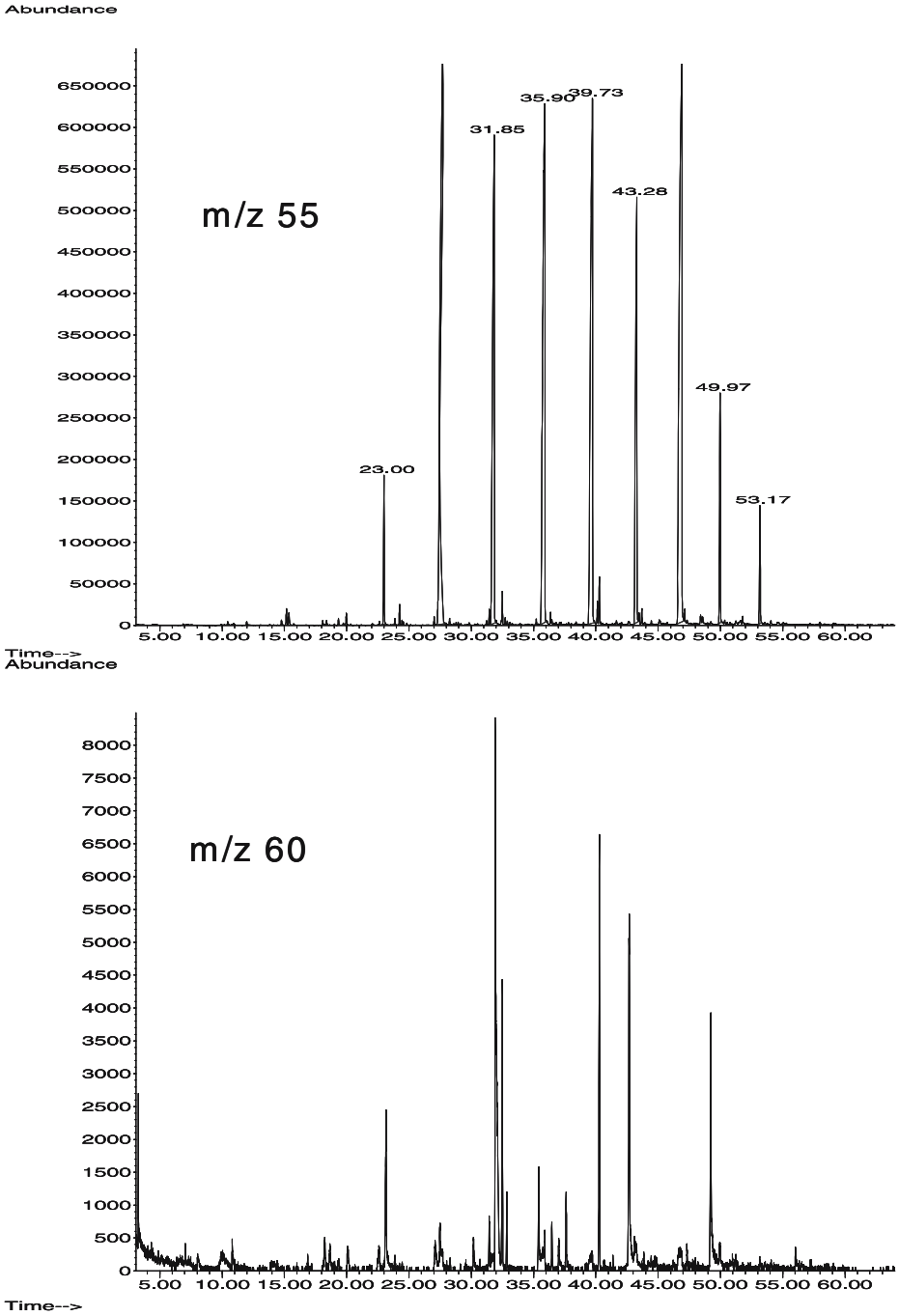


Fig. 2 Simplified selected-ion chromatograms (SICs) for extracted ions at m/z 55 and m/z 60 in a representative preen oil sample

through qualitative and quantitative comparisons. Table 1 lists the identified compounds together with their retention times and statistical significance classified by both season and sex. The compounds found prominently in preen oil samples included a homologous series of linear alcohols (C_9–C_{18}) as the major components in all samples (peak areas 100–1,000

Table 1 Compounds used for quantitative comparisons between breeding and nonbreeding conditions

	Compound	R_t (min)	TIC or Ion m/z	Female *P**	Male *P**
1	Octanal	11.05	55		
2	p-Cymene	11.98	TIC		
3	2-Ethyl-1-hexanol	12.00	TIC		
4	Undecane	15.28	TIC		
5	Nonanal	15.47	55		
6	1-Nonanol	18.62	55		
7	Decanal	20.17	55		
8	Nonanoic acid	22.90	60		
9	1-Decanol	23.20	55	0.01	0.004
10	Tridecane	24.39	TIC		
11	2-Undecanone	24.04	58		
12	Undecanal	24.66	TIC		
13	Decanoic acid	27.10	60		
14	1-Undecanol	27.87	55	0.01	0.002
15	Geranylacetone	30.55	TIC		
	Internal standard (added)	31.44	113		
16	1-Dodecanol	32.00	55	0.004	0.002
17	2-Tridecanone	32.69	58		0.002
18	Dodecanoic acid	35.16	60	0.028	
19	1-Tridecanol	36.00	55	0.16	0.002
20	2-Tetradecanone	36.30	58		0.002
21	1-Tetradecanol	39.82	55	0.028	0.002
22	2-Pentadecanone	40.49	58		0.004
23	Heptadecane	40.58	TIC		
24	3-Methyltridecanoic acid	40.79	TIC		
25	Tetradecanoic acid	42.73	60		0.044
26	1-Pentadecanol	43.44	55	0.01	0.002
27	A C18 alkene	43.63	TIC		
28	2-Hexadecanone	43.72	58		
29	1-Octadecene	43.87	TIC		
30	3-Methyltetradecanoic acid	44.34	TIC		
31	Hexadecanal	44.65	TIC		
32	An alkene	45.39	TIC		
33	1-Hexadecanol	46.95	55	0.016	0.002
34	2-Heptadecanone	47.15	58		
35	3-Methylpentadecanoic acid	47.65	TIC		
36	A C20 alkene	48.77	TIC		
37	Hexadecanoic acid	49.15	60	0.044	0.010
38	1-Heptadecanol	50.22	55		0.002
39	1-Octadecanol	53.50	55	0.044	0.002

*P**: Mann–Whitney *U* test (two-tailed) for significant seasonal differences for female (N=6) and male (N=6) matched pairs

times larger than minor compounds). Additionally, the series of aldehydes, acids, and methylketones were included in such volatile profiles. Among the minor components, some branched acids and saturated and unsaturated hydrocarbons were notably detected (see also Fig. 1).

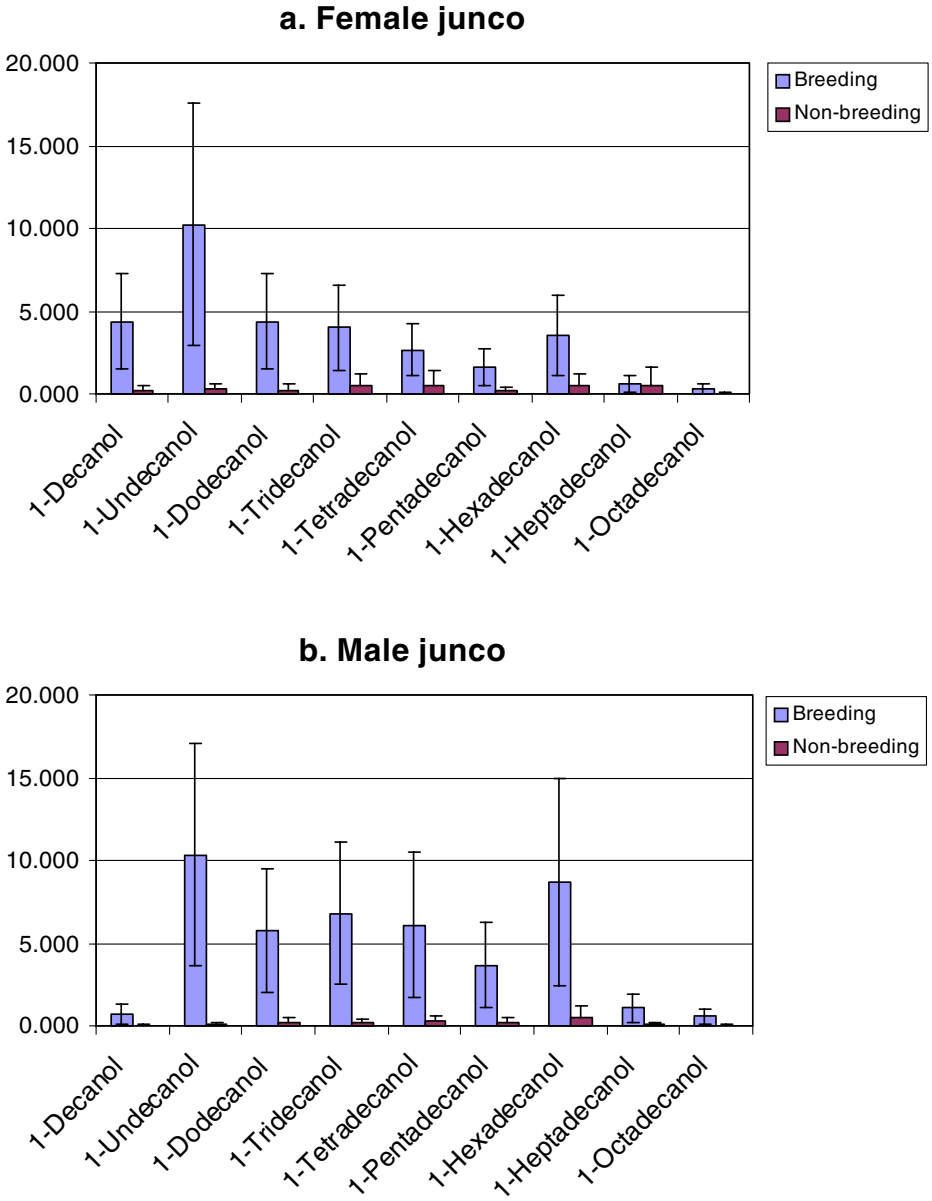


Fig. 3 (a) Averages of the normalized peak areas of C_{10}–C_{18} alcohols in female junco preen oil samples (N=6) in breeding and nonbreeding conditions. Error bars indicate individual variation (standard deviation, SD). (b) Averages of the normalized peak areas of C_{10}–C_{18} alcohols in male junco preen oil samples (N=6) in breeding and nonbreeding conditions. Error bars indicate individual variations (standard deviation, SD)

Linear Alcohols The highest levels of *n*-alkanols were detected as C_{10}–C_{18} (from decanol to octadecanol). Also, 1-nonanol, was detected at lower concentrations. Using the extracted ion profiles for *m/z* 55 (see Fig. 2), the peak areas of alcohols were measured and normalized with the peak area of the internal standard (*m/z* 113). In Fig. 3, we show the plotted normalized peak areas for the major alcohols of female and male juncos, respectively. In the breeding condition, as compared to the nonbreeding condition, both junco sexes showed elevated levels of these alcohols, although their individual variations appeared relatively large, as indicated by the error bars in Fig. 3.

Except for 1-heptanol in the female group, all *n*-alcohols, from 1-decanol to 1-octadecanol, showed statistically significant seasonal increases ($P<0.05$) in both males and females under long days. As seen in Table 1, based on their smaller *P* values, the differences tended to be larger in males than females. The compound 1-decanol was an exception in that it appeared more elevated in breeding females than in breeding males, but the sex difference was only marginally significant ($P<0.052$). During the nonbreeding season, concentrations of the linear alcohols did not differ between females and males.

Linear *n*-alcohols, such as those seen here as the major volatile components in the preen oil of the dark-eyed junco, have been reported as effective natural antimicrobial and antifungal substances for a wide spectrum of gram-positive bacteria, molds, and yeasts (Kubo et al., 1993, 1995, 2003). Indeed, these alcohols could have multiple functions during a junco breeding season as antimicrobial and antifungal agents. Juncos are high-latitude and high-altitude birds, and during winter their environments tend to be cold enough to suppress growth of both bacteria and fungi. However, warmer temperatures increase microbial growth, and alcohols may be a response to increased exposure, serving to promote healthy feathers and conditions at the nest.

In addition, plant leaves typically contain linear alcohols (Vioque and Kolattukudy, 1997). Increased levels of *n*-alcohols in preen oil during summer may help both female and

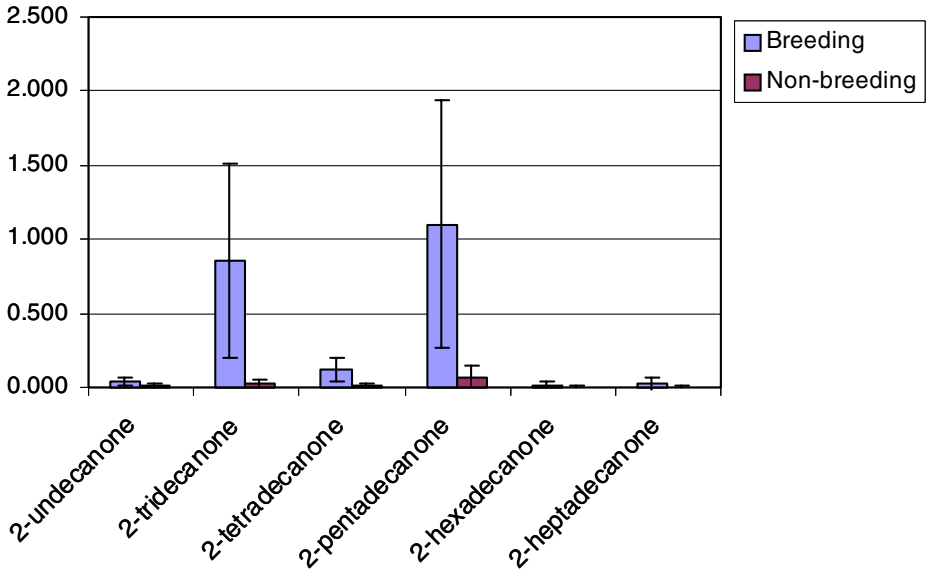


Fig. 4 Averages of the normalized peak areas of methylketones in male junco preen oil samples ($N=6$) in breeding and nonbreeding conditions. Error bars indicate individual variations (standard deviation, SD)

male juncos to blend their odors with the background odors of the plants in their environment (odor blending), for example, when the female with incubating eggs in a ground nest or the male is approaching the nest with food. Junco's eggs and young are a common prey of mammalian predators such as mice, chipmunks, and raccoons, so odor camouflage could provide a significant advantage (Nolan et al., 2002; Clotfelter et al., 2004).

Linear Methylketones A pattern of linear methylketones was seen in all preen gland samples (both male and female juncos). Comparing their peak areas to those of *n*-alkanols, methylketones were minor components. The peak areas for these compounds were measured using the extracted ion at *m/z* 58.

2-Tridecanone and 2-pentadecanone levels were higher than the remaining 2-ketones for the breeding male juncos, as shown in Fig. 4. A statistically significant increase (compared to nonbreeding season), was found for 2-tridecanone ($P<0.002$), 2-tetradecanone ($P<0.002$), and 2-pentadecanone ($P<0.004$) (see also Table 1). Female juncos showed an increase during the breeding season for 2-tridecanone and 2-pentadecanone levels, although these levels were somewhat lower than in males, and the changes were only close to statistical significance as a result of the large individual variations ($P<0.051$). A comparison between the male and female levels of 2-ketones showed only slight tendency for higher 2-pentadecanone levels in breeding males vs. breeding females ($P<0.186$).

Methylketones are also commonly found components in mammalian epidermis, including human skin (Penn et al., 2006). In birds, similarly to *n*-alcohols, methylketones may be promoting healthy feather conditions by acting as chemical defense compounds. Methylketone activity has previously been shown to provide plants with protection from herbivores and fungal pathogens, having been reported as a chemical defense compound in tree bark (McDowell et al., 1988), and as protection against the spider mite in wild and cultivated tomato plants (Chatzivasileiadis et al., 1999). Perhaps they provide similar protection for feathers.

Fatty Acids Preen samples contained linear and branched carboxylic acids at relatively low levels. The linear acid concentrations were higher than for the branched acids. The linear-

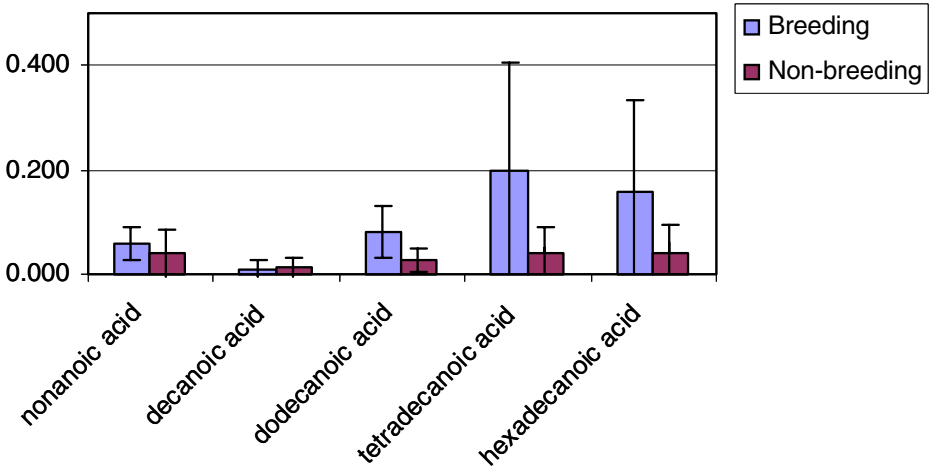


Fig. 5 Averages of the normalized peak areas of linear acids (C_9, C_{10}, C_{12}, C_{14}, C_{16}) in female junco preen oil samples ($N=6$) in breeding and nonbreeding conditions. Error bars indicate individual variations (standard deviation, SD)

acid peak areas were measured by using the extracted ion at *m/z* 60. Individual variation within all acid concentrations in the samples was quite large, as shown in Fig. 5, for the female juncos. Moreover, for this class of compounds, the differences between the breeding and nonbreeding conditions were not clear. However, in both female and male junco samples, tetradecanoic and hexadecanoic acid (even number fatty acids) levels were elevated more than for similar constituents (shown in Fig. 5) in the breeding condition. In both sexes, a statistically significant increase during breeding was found only for hexadecanoic acid ($P<0.044$ for females and $P<0.010$ for males). Additionally, the seasonal increase in dodecanoic acid was significant: for females, dodecanoic acid ($P<0.028$) and for males tetradecanoic acid ($P<0.044$). However, the acid concentrations did not differ statistically between females and males during the seasons.

Branched fatty acids were detected at lower levels than linear fatty acids, but also with large individual variations (data not shown). Their levels did not differ significantly by season in either male or female juncos.

Human sebum and circulating lipids contain a large number of different linear and branched fatty acids. It was suggested long ago that the ratios of the acidic constituents in mammalian skin lipids may carry an olfactory signature of an individual (Nicolaides, 1974). Interestingly, mammalian wax monoester biosynthesis involves fatty acids as starting materials, followed by reduction to linear alcohols (fatty alcohols) by a specific reductase enzyme. A wax synthase enzyme converts fatty alcohols finally to wax monoesters (Cheng and Russell, 2004a, b). Similarly, wax ester synthesis via linear alcohols was suggested for algae even earlier (Kolattukudy, 1970).

In our current study, the levels of nonvolatile wax monoesters were not of a direct interest. Whether their levels vary seasonally in the preen oil is not currently known. However, although fatty acid compositions were variable among the individuals across the seasons, the levels of linear alcohols (possibly fatty acid reduction products) increased dramatically. Accordingly, one might postulate that the wax ester synthesis route may slow down during the breeding season, yielding more *n*-alcohols as fatty acid reduction products.

One of the more interesting implications of volatile preen gland secretions is that the odors they create could serve to signal identity, condition, relatedness, or other information about an individual. This information might be utilized by conspecifics to make an assessment of the signaler. Compounds with large individual variation may be particular candidates as signals. The MHC gene products, for example, have been hypothesized both to facilitate immune responses (Apanius et al., 1997, Penn and Potts, 1999) and to produce recognizable odors (Penn and Potts, 1998, Spehr et al., 2006). Furthermore, some studies have speculated that these odors might be used to promote adaptive mate choice, as they allow individuals to assess their relatedness and regulate their degree of inbreeding (Potts and Wakeland, 1990; Brown and Eklund, 1994; Penn and Potts, 1999).

Interestingly, *CD1* genes (evolutionary predecessors of the MHC genes) which have been found in humans, have also been identified in birds. Correspondingly, these genes code for proteins that present antigens such as fatty acids and glycolipids to T cells, rather than the more typical presentation of peptides (Miller et al., 2005; Salomonsen et al., 2005). These genes might also play a role in regulating similar fatty acid compositions as found in preen gland secretions (Porcelli and Modlin, 1999). Enhanced detection of variation in the *MHC* or *CD1* genes might also be useful for dark-eyed juncos, particularly for those that breed in the Appalachian Mountains where natal philopatry is high (Nolan et al., 2002) and thus the potential for inbreeding is also high. Individuals able to detect variation at the MHC and, possibly CD1 loci, could potentially benefit from inbreeding avoidance and increased offspring fitness by maintaining immunogenetically important MHC or CD1 heterozygosity.

Comparisons of the other compounds, which appeared at low levels (e.g., *p*-cymene, saturated and unsaturated hydrocarbons, and some aldehydes) did not result in clear differences by sex or between the breeding and nonbreeding conditions (data not shown).

Sulfur Compound Profiles Several individuals from each group of breeding and nonbreeding males and females were qualitatively screened using the ultrasensitive sulfur compound detection (GC-AED). The sorptive stir bar extraction method has been previously shown to recover efficiently sulfur compounds at picogram quantities from biological samples (Soini et al., 2005). In this study, the breeding males consistently showed a higher number of low (picogram level) sulfur compounds than nonbreeding males and females (breeding or nonbreeding). No identifications could be made because of the low levels detected in the sulfur detection mode (data not shown).

Volatile Compound Profiles on the Feather Surface Among the volatile compounds collected from the wing surface were several compounds, including *n*-alcohols, which were obviously shown as originating from the preen oil (see Fig. 6). Additionally, numerous compounds not found previously in preen oil were detected on the feather surface. These compounds may be originating from the bird epidermal lipids (Menon and Menon, 2000), their surface-catalyzed conversions, or environmental sources. A large porous surface area on feathers is known to be efficient in enriching airborne particulates, including metals. Several studies have utilized bird feather surface for a biological monitoring of environmental heavy metal pollutants (for review see, Burger, 1994; Veerle et al., 2004).

In summary, the volatile compounds found in *J. hyemalis* preen gland secretions were found to have increased during the breeding season in both sexes, and the seasonal variations may reflect a number of possible functions for these components including defense against bacteria and fungi, odor camouflage in relation to nest predators, or information to

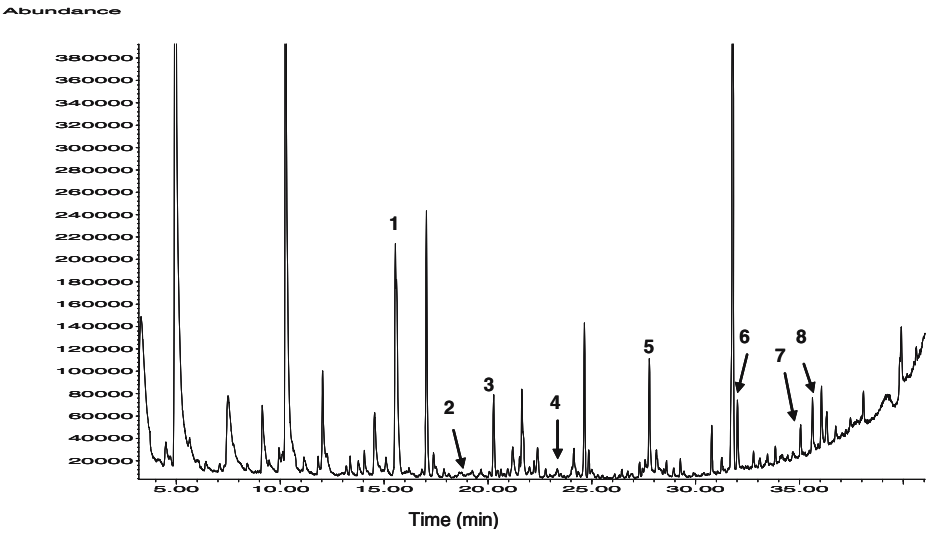


Fig. 6 A volatile compound GC–MS TIC profile collected directly from the surface of the male junco wing feather. Compounds, which were also found in the preen oil, are marked by numbers 1: nonanal, 2: 1-nonanol, 3: decanal, 4: 1-decanol, 5: 1-undecanol, 6: 1-dodecanol, 7: dodecanoic acid, 8: 1-tridecanol

be communicated to conspecifics, such as chemosignals about condition (appearance) or genotype (degree of relatedness). A large individual variation in the volatile compound levels could even indicate their regulation by *MHC* or *CD1* genes. More experiments are needed to test possible functions of the linear alcohols, methylketones, and linear carboxylic acids whose concentration increased during the breeding. Such experiments will require a carefully controlled design of behavioral and hormonal conditions.

Acknowledgments This work was jointly sponsored by the METACyt Initiative at Indiana University, and the Lilly Chemistry Alumni Chair funds (to M.V.N.) and the National Science Foundation grant NSF BSC 05-19211, 2005–2008 (to E.D.K.). We thank Mr. Michael Wigen for technical assistance.

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J Chem Ecol (2007) 33:199–212
DOI 10.1007/s10886-006-9204-y

More Rare Males in *Ostrinia*: Response of Asian Corn Borer Moths to the Sex Pheromone of the European Corn Borer

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Received: 21 June 2006 / Revised: 5 September 2006 / Accepted: 12 September 2006 /
Published online: 5 December 2006
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Abstract A previous flight tunnel study showed that 3–5% of European corn borer (ECB) moths, *Ostrinia nubilalis* (*Z*/*E*11-14:OAc), could fly upwind and make contact with sources releasing the sex pheromone of the related Asian corn borer (ACB), *Ostrinia furnacalis* (2:1 *Z*/*E*12-14:OAc). In this study, we show that rare males (3–4%) are also present in South Korean ACB that respond to the sex pheromone blends of the ECB UZ (97:3 *Z*/*E*11-14:OAc) and BE (1:99 *Z*/*E*11-14:OAc) pheromone races. We also show that the upwind flight response of a significant proportion of male ACB was antagonized by the addition of 1% *Z*9-14:OAc to the ACB blend, a compound that also antagonizes the upwind flight of ECB males. Male ACB flight behavior was not, however, affected by adding either of the ECB blends to the ACB blend, or by the addition of 50% 14:OAc, a compound identified from female pheromone glands of ACB and a number of other *Ostrinia* species. Additional flight tunnel tests with ACB to study the comparative aspects of ECB and ACB pheromone response specificity showed that male ACB exhibited maximal levels of upwind flight and source contact with doses of pheromone (30 and 100 μg on rubber septum sources) that also elicited maximal levels in the two ECB pheromone races. The maximal level of source contact for ACB (66%) was lower than observed with the UZ race of ECB to its pheromone blend (>95%), but comparable to those for the BE race of ECB (65–70%). Male ACB also flew upwind in high proportions to a broader range of ratios of *Z*/*E*12-14:OAc (80:20 to 20:80) than was previously observed for either of the ECB races.

Keywords Asian corn borer · Behavioral antagonist · European corn borer · Flight tunnel

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Ostrinia furnacalis · *Ostrinia nubilalis* · Pheromone evolution · Response specificity · Sex pheromone

Introduction

The genus *Ostrinia* (Lepidoptera: Crambidae) is composed of 20 species, including two of agricultural importance, the European corn borer (ECB), *Ostrinia nubilalis* (Hübner), and the Asian corn borer (ACB), *Ostrinia furnacalis*. The ECB has been studied more extensively and exists in Europe and North America as two distinct pheromone races (Kochansky et al., 1975; Roelofs et al., 1985). Most populations (Z race) use a 97:3 blend of (*Z*)-11- and (*E*)-11-tetradecenyl acetates (*Z*11/*E*11-14:OAc), but occur in some areas sympatrically with an E race that uses the opposite 1:99 *Z*/*E* blend. In New York state, three different races of ECB, based on the sex pheromone blend and the number of generations (voltinism), have been described: bivoltine Z (designated BZ), univoltine Z (UZ), and bivoltine E (BE) (Roelofs et al., 1985). Studies of the genetic basis of the ECB pheromone system have shown that there are three genes responsible for the differences (Roelofs et al., 1987; Löfstedt et al., 1989; Cossé et al., 1995).

The ACB, which occupies the ecological niche in Asia equivalent to that occupied by ECB in Europe (Ishikawa et al., 1999a), uses mixtures of (*Z*)-12- and (*E*)-12-tetradecenyl acetates (*Z*12/*E*12-14:OAc) that vary from 1:1 in the Philippines, mainland China, Taiwan, and some parts of Japan (Klun et al., 1980; Cheng et al., 1981; Du et al., 1986; Kou et al., 1992) to 2:1 in the southernmost part of Japan (Ando et al., 1980; Huang et al., 1998) and South Korea (Boo and Park, 1998).

In studies on the desaturase enzymes involved in controlling pheromone biosynthesis, Roelofs et al. (2002) discovered that the female pheromone gland of the ECB contains the expected desaturase gene that makes Δ11-14:Acid for production of its pheromone components, but the gland also contains the gene for a Δ14-16:Acid desaturase used by the ACB. The ACB pheromone gland likewise contains the same two desaturase genes, but only expresses the Δ14 desaturase, which it uses for its pheromone components. Roelofs et al. (2002) proposed that a saltational event occurred in an ancestral *Ostrinia* moth population and gave rise to a new pheromone and new species, the ACB. A possible pseudogene that had been unexpressed for millions of years in the genome of *Ostrinia* became functional and generated pheromone precursor fatty acids that differed in double-bond position and chain length. The resulting pheromone acetates then possessed a double bond in the Δ12 position instead of the Δ11 position. The desaturase studies raised the possibility that a new pheromone communication system can arise as a result of changes in regulatory pathways controlling gene expression. However, for the new pheromone to be effective in mate location it is necessary for some males to be able to detect and respond to the new blend. Subsequent flight tunnel studies showed that some males (3–5%) in the UZ and BE *O. nubilalis* races did, in fact, respond to both species' pheromone blends (Linn et al., 2003).

In this study, we report the results of flight tunnel experiments showing that there also are rare ACB males from a population originating in South Korea that respond to one or both of the ECB blends. We show further that the upwind flight of ACB males to their pheromone is antagonized by the addition of *Z*9-14:OAc, as it occurs with ECB, but not by the addition of the ECB blends to the ACB mixture. We also provide support for the claim that tetradecyl acetate (14:OAc), although present in the pheromone glands of many *Ostrinia* species, including ACB, does not enhance or disrupt levels of upwind flight.

Methods and Materials

Insects

Colonies of the UZ and BE races of *O. nubilalis* from New York state were maintained in separate walk-in environmental chambers. Mating and larval rearing conditions were as described in Roelofs et al. (1987) at a constant temperature of 25°C and 16:8 L/D photoperiod. Pupae were sexed and the male pupae were placed on a layer of vermiculite in plastic and screen emergence cages inside a walk-in environmental chamber (25°C, 16:8 L/D photoperiod). Cages of adults were separated daily so that individuals of known age could be used for flight tunnel tests.

The ACB were obtained from Jin Kyo Jung, National Institute of Crop Sciences, South Korea, and maintained on the same diet and environmental conditions in the lab in New York as were the ECB populations.

Chemicals

*Z*11-14:OAc, *E*11-14:OAc, *Z*12-14:OAc, *E*12-14:OAc, *Z*9-14:OAc, and 14:OAc were obtained from the Pherobank (http://www.pherobank.nl), and mixtures were prepared in high-performance liquid chromatography grade hexane and applied to red rubber septa (Thomas Scientific, Swedesboro, NJ, USA; Glover et al., 1989; Linn et al., 1997).

Flight Tunnel

Adults were tested in the sustained flight tunnel during their second to third night after ecolosion under standard conditions for *O. nubilalis* (Glover et al., 1989; Linn et al., 1997): 20–21°C, 60–65% RH, 0.50 m/sec air flow, and illumination of 11 lux of red light at the tunnel floor during the third to sixth hour of scotophase. Adults were taken to the room housing the flight tunnel 1 hr before the start of the eighth hour of scotophase and placed individually in screen release cages so they could acclimate to the flight tunnel room environment. The temperature during the 1-hr period of acclimation, in photophase, was 25°C, then, when lights off occurred the temperature dropped to 20–21°C. Adult moths were tested individually, and a positive response was counted if the male exhibited upwind flight in the odor plume and made contact with the rubber septum source.

Six experiments were conducted, each designed to address a different question about the comparative response specificity of ACB and ECB males. In some cases, individual males were tested to more than one treatment during each test period. After recapture, males were removed from the flight tunnel and allowed a minimum period of 15 min before retesting to a different blend. Previous experiments have demonstrated that ECB can be tested up to four times in a daily testing period without adverse affects on performance (Cossé et al., 1995). Preliminary experiments indicated that ACB could also be tested three times during a single testing period with no adverse affects on levels of upwind flight.

Experiment 1: Response of ACB Males to a Dose Series of Pheromone

ACB males were tested to a dose series of the 2:1 *Z*/*E*12-14:OAc blend: 1, 3, 30, 100, and 300 μg/septum. During each testing period, a minimum of 10 ACB males were tested to at

least two of the five doses, with each dose tested a minimum of four different testing periods (days).

Experiment 2: Response of ACB Males to Different Blends of *Z*/*E*12-14:OAc

ACB males were tested to 30-μg doses of the following *Z*/*E*12-14:OAc blends: 4:1, 2:1, 1:1, 1:2, and 1:4. During each day of testing, a minimum of 10 males were tested to three of the blends, with treatment blends rotated over subsequent days to achieve approximately the same number of males tested to each blend.

Experiment 3: Are There Rare ACB Males that Respond to the ECB Blends?

Two protocols were used for this experiment. In the first, ACB males and UZ race ECB males were tested to a 30-μg dose of the 2:1 *Z*/*E*12-14:OAc blend (ACB blend) and to a 30-μg dose of the 97:3 *Z*/*E*11-14:OAc UZ ECB blend (UZ blend). Males of each species were tested during each daily test period, beginning with the ACB blend for UZ males and the UZ blend for ACB males. In the second protocol, ACB males were tested to 30-μg doses of the UZ blend, 1:99 *Z*/*E*11-14:OAc BE ECB blend (BE blend), and the ACB blend. Each male was tested sequentially to the three blends in the same testing period, beginning with either of the ECB blends and ending with the ACB blend.

Experiment 4: Effect of *Z*9-14:OAc on Male ACB Response to the ACB Pheromone Blend

ACB males were tested to a 30-μg dose of the ACB blend alone, or with 1% *Z*9-14:OAc added to the mixture. The 1% value was chosen based on previous studies (Glover et al., 1989). For comparison, male ECB of the BE race were also tested on each day to a 30-μg dose of the BE blend alone, or with 1% *Z*9-14:OAc added to the mixture.

Experiment 5: Response of ACB Males to the Combined ACB/ECB Blends

ACB males were tested to a 30-μg dose of the ACB blend alone or with a 30-μg dose of the UZ or BE blends added.

Experiment 6: Response of ACB Males to the ACB Blend with 14:OAc Added

Previous studies with ACB have shown that 14:OAc is present in female pheromone glands, but has never been shown to enhance trap capture of moths (Boo and Park, 1998). In our experiment, ACB males were tested to a 30-μg dose of the ACB blend alone, or with 50% of the compound 14:OAc added. The 50% value was relative to the amount of *Z*12-14:OAc in the blend and was selected based on the study by Boo and Park (1998) showing that female glands from 2- to 3-d-old females contained a mean ratio of 0.74:1:1.36 for 14:OAc, *E*12-14:OAc, and *Z*12-14:OAc, respectively.

Statistical Analysis

For each experiment the number of moths in each treatment that exhibited upwind flight to the source was combined for all testing periods and converted to a percent for graphical display.

Chi-square analysis or post hoc pairwise comparisons using Fisher's exact tests were implemented according to the JMP statistical analysis program for Macintosh $P<0.05$).

Results

Experiment 1: Response of ACB Males to a Dose Series of Pheromone

The greatest percentage of source contacts by ACB males occurred with the 30-μg dose of the ACB blend (66%, $N=40$; Fig. 1a). This value was not significantly different from the lower percentages with the 3-μg (53%, $N=53$; Fisher's exact test $P=0.273$, $df=1$) and 100-μg (63%, $N=44$; Fisher's exact test $P=0.825$, $df=1$) doses. However, with the 1-μg

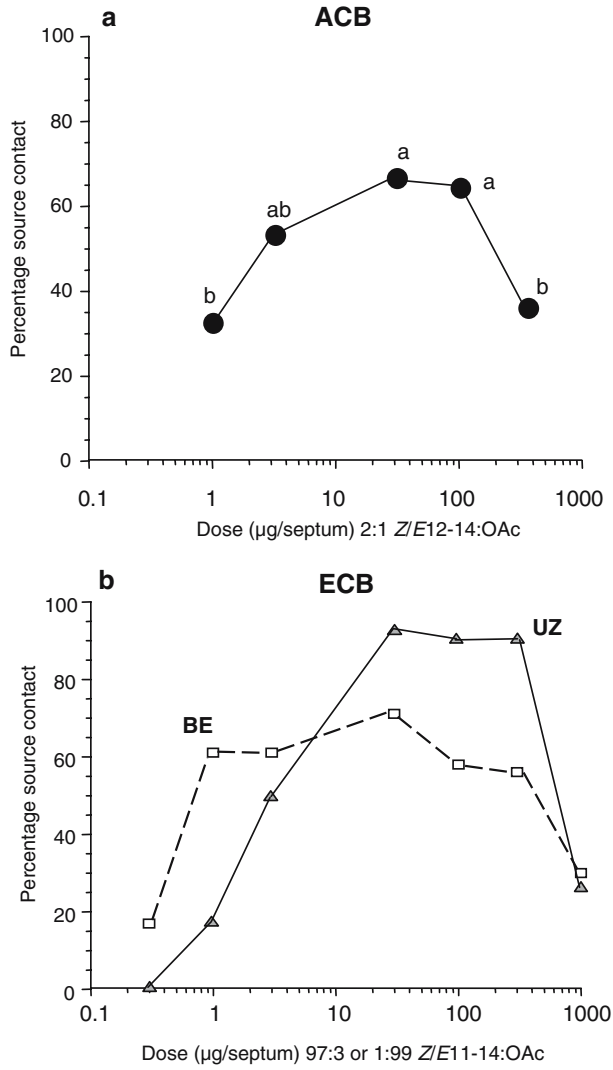


Fig. 1 Percentage source contact by ACB males tested to a dose series (1, 3, 30, 100, and 300 μg; $N=63$, 64, 60, 65, and 62, respectively) of the 2:1 *Z*/*E*12-14:OAc ACB pheromone (**a**). Data points with different letters are significantly different ($P<0.05$) according to selected post hoc Fisher's exact tests (see text). For comparison, response values for ECB males (UZ males to 97:3 and BE males to 1:99 *Z*/*E*-11-14:OAc) are presented in subpanel **b**, modified from Linn et al. (1997)

(32%, N=43) and 300-μg (35%, N=42) doses, there were significant reductions in the percentages of source contacts compared with the 100-μg dose (Fisher's exact test P= 0.004, df=1 for 3 μg vs. 100 μg). Based on these results, the 30-μg dose of the ACB blend was used for the other experiments. Previous results with two populations of ECB also showed that 30-μg of the 97:3 or 1:99 Z/$E$11-14:OAc blends elicited maximal response levels (Fig. 1b; modified from Linn et al., 1997; see also Glover et al., 1989). Maximal response levels were lower for the BE (72%, 30 μg) and ACB (66%, 30 μg) populations than for UZ males (93%, 30 μg) (Fig. 1b).

Experiment 2: Response of ACB Males to Different Blends of Z/$E$12-14:OAc

There were no significant differences in the response levels of ACB males to the 30-μg dose of five ratios of Z/$E$12-14:OAc (Fig. 2a; χ^2=5.296, P>0.05). Interestingly, ACB

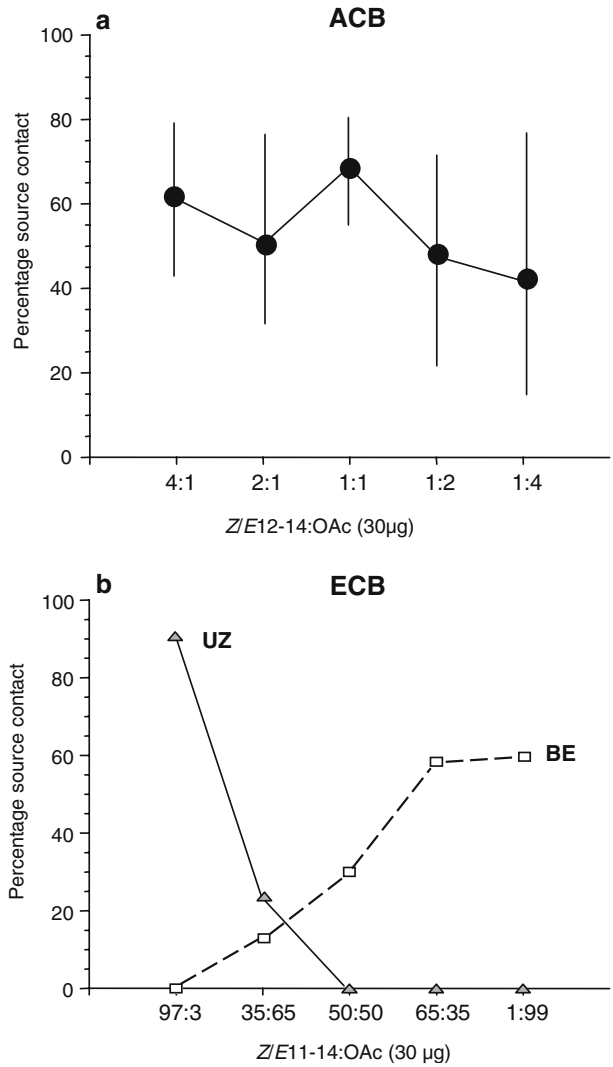


Fig. 2 Percentage source contact by ACB males tested to 30-μg doses of five different ratios (4:1, 2:1, 1:1, 1:2, and 1:4 Z/E; N=63, 67, 64, 67, and 62, respectively) of the ACB pheromone (**a**). There were no significant differences in the response values (χ^2, P>0.05), and lines above and below data points indicate the range of values obtained over 6 d of testing. For comparison, response values for UZ and BE race, ECB males to different ratios of Z:$E$11-14:OAc are presented in subpanel **b**, modified from Linn et al. (1997)

males flew upwind in the greatest percentage to the 1:1 *Z*/*E*12-14:OAc ratio (69%), and not the 2:1 mixture that occurs in the glands (Boo and Park, 1998). The measurement of 50% for source contacts with the 2:1 ratio in this experiment was the lowest measurement for that blend in all of the other experiments (generally in the 60–70% range, see below). For most of the ratios, there was considerable variability in the male preference for blend quality over the 6-d testing period (Fig. 2a).

Comparative data from Linn et al. (1997) for the upwind flight response of UZ and BE ECB males to a range of *Z*/*E*11-14:OAc ratios show that ACB males displayed a much lower preference for odor quality than did ECB males (Fig. 2b). UZ males displayed a high specificity for the 97:3 *Z*/*E*11-14:OAc blend that occurs in the glands, with >90% reaching the source, but only 24% flying upwind to a 35:65 *Z*/*E* ratio, and none to the 50:50 blend. Although BE males also displayed a preference for the identified 1:99 *Z*/*E*11-14:OAc blend, they also reached the source in approximately equivalent proportions with a 65:35 *Z*/*E*11-14:OAc mix, and 12% of the tested males exhibited complete flights to a source, releasing a 35:65 *Z*/*E*11-14:OAc mix. The combined results from experiment 2 and previous studies of ECB suggest that with respect to specificity for odor quantity and quality, the three populations can be ranked: UZ > BE > ACB.

Experiment 3: Are There Rare ACB Males that Respond to the ECB Blends?

Rare males were found in the ACB population that exhibited complete flights to the UZ ECB pheromone source (30 μg, Fig. 3). Of 525 ACB males tested to the UZ blend, 3%

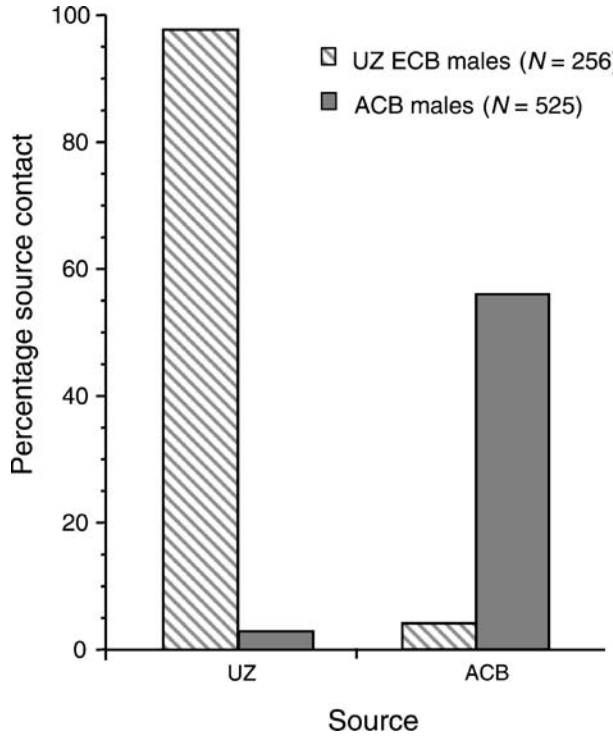


Fig. 3 Percentage source contact of male UZ ECB and male ACB to the 30-μg source of the UZ (97:3 *Z*/*E*11-14:OAc) and 30-μg source of the ACB (2:1 *Z*/*E*12-14:OAc) pheromone blends. For UZ ECB, 256 males were tested to the ACB blend, with 4% (*N*=10) reaching the source. Of the UZ males that did not fly upwind to the ACB blend 213 were then tested to the UZ blend, with 97% (*N*=207) reaching the source. For ACB, 525 males were tested to the UZ blend, with 3% (*N*=16) reaching the source. Of the ACB males that did not fly upwind to the UZ blend 250 were then tested to the ACB blend, with 56% (*N*=140) reaching the source

(16 males) made source contact. These same 16 males also flew upwind to the 30-μg dose of the ACB blend. Additionally, of the 509 ACB males tested to the UZ blend that did not fly upwind to that source, 250 were also tested to the ACB blend, with 56% (N=140) making source contact.

For UZ males, 256 were tested to the 30-μg dose of the ACB blend, with 4% (10 males) reaching the source (Fig. 3). These same 10 males also flew upwind to the 30-μg dose of the UZ blend. Additionally, of the UZ males tested to the ACB blend that did not fly upwind to that source, 213 were also tested to the UZ blend, with 97% (N=207) making source contact.

In a second testing protocol to detect the presence of rare ECB blend-responding ACB males, ACB males were tested to 30-μg doses of the UZ and BE blends, and to 30 μg of the ACB blend. Of 460 males tested, 61% (N=281) made contact with the ACB source; 4% (N=18) reached the UZ and ACB sources; 3% (N=14) reached the BE and ACB sources; and 1% (N=5) flew upwind to all three sources (Fig. 4).

Experiment 4: Effect of *Z*9-14:OAc on Male ACB Response to the ACB Pheromone Blend

The percentage of ACB males flying upwind to the 30-μg dose of the ACB blend was reduced when 1% *Z*9-14:OAc was added to the ACB blend (Fig. 5; Fisher's exact test $P<0.001$, df=1). With the ACB blend alone, 66% (N=129) of the males reached the source, but only 30% did so when 1% *Z*9-14:OAc was added. In comparison, 77% of ECB males from the BE colony reached the source in response to the BE blend (30 μg) alone, but only

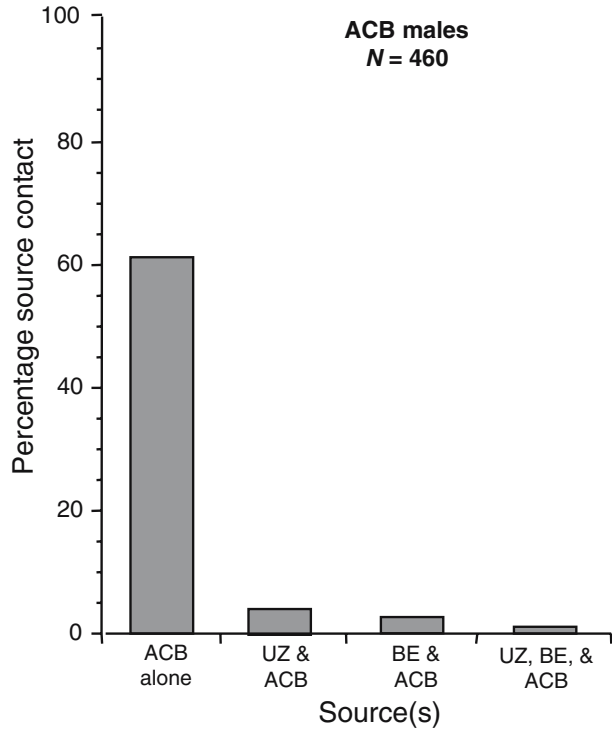


Fig. 4 Percentage source contact by male ACB (N=460) tested in sequence to the BE (1:99 *Z*/*E*11-14:OAc; 30 μg), UZ (97:3 *Z*/*E*11-14:OAc; 30 μg), and ACB (2:1 *Z*/*E*12-14:OAc; 30 μg) pheromone blends. Response values show percentage of males reaching the source of the ACB blend alone (61%, N=281), those reaching the ACB and UZ sources (4%, N=18); those reaching the BE and ACB sources (3%, N=14); and those flying upwind to all three blends (1%, N=5). None of the ACB males flew upwind to one or both of the ECB blends, but not to ACB blend

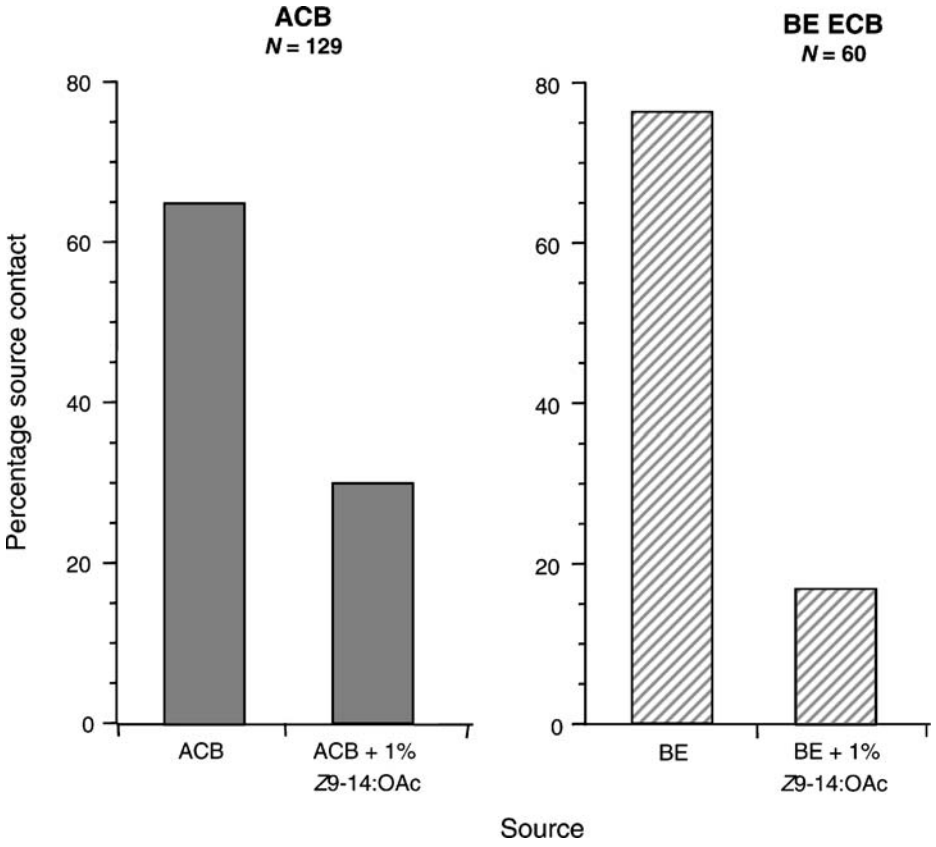


Fig. 5 Percentage ACB (N=129) and BE ECB (N=60) making source contact with the 30-μg ACB (2:1 *Z*/*E*12-14:OAc) or the BE (1:99 *Z*/*E*11-14:OAc) blend and then to the ACB blend with 1% *Z*9-14:OAc added, or the BE blend with 1% *Z*9-14:OAc added

17% did so when 1% *Z*9-14:OAc was added to the blend (Fig. 5; Fisher's exact test $P<0.001$, $df=1$).

Experiment 5: Response of ACB Males to the Combined ACB/ECB Blends

The upwind flight response of ACB males to a 30-μg dose of the ACB blend was unaffected by the addition of the UZ or BE blend (30 μg). With the ACB blend alone, 66% of the tested males (N=64) reached the source, and when the UZ or BE blend was added 68% (N=60; Fisher's exact test $P=0.894$, $df=1$) and 57% (N=65; Fisher's exact test $P=0.368$, $df=1$) of ACB males, respectively, reached the source.

Experiment 6: Response of ACB Males to the ACB Blend with 14:OAc Added

The upwind flight response of ACB males to the ACB blend also was unaffected by the addition of 14:OAc. With the 30-μg dose of the ACB blend alone, 64% of the tested males

(N=55) reached the source, and when 50% 14:OAc was added to the ACB blend, 60% (N=50; Fisher's exact test P=1.000, df=1) reached the source.

Discussion

Our results provide several important findings concerning the response specificity of ACB males for their pheromone, and allow comparisons with previously published results for the closely related ECB. First, there are rare ACB males that respond to their own pheromone and to one or both of the ECB blends. Second, the upwind flight response of ACB males was antagonized by the addition of *Z*9-14:OAc to the pheromone blend, similar to ECB, but was unaffected by addition of either of the ECB blends. Third, ACB males responded in high proportions to a wider range of blend ratios than did ECB males. Fourth, addition of 14:OAc, a previously identified compound in ACB pheromone glands, did not affect the upwind flight response of ACB males to the 2:1 *Z*/*E*12-14:OAc blend.

Rare ACB Males Responding to the ECB Pheromone Blends

Male ACB were found that responded to the ECB UZ blend (4%), the BE blend (3%), and to both the UZ and BE blends (1%), in addition to the ACB blend. The upwind flight percentages are comparable with levels of 3–5% for ECB males of the UZ and BE races that responded to the ACB blend (Linn et al., 2003). The finding that some ACB males can detect and respond to the ECB pheromone blends adds to a growing list of cases involving the presence of broadly responsive individuals in moth populations (Baker, 2002) that have the capacity to process information about pheromone components utilized by related species in an agonistic manner. The adaptive value of being a broad responder is that these individuals have the potential to exploit new communication channels, as has been proposed for the ACB (Baker, 2002; Roelofs et al., 2002). The finding also is not entirely unexpected given the proposed phylogenetic relationships in the *Ostrinia* (Ishikawa et al., 1999a, b). Most of the *Ostrinia* species in Asia, from which the ACB were derived (Roelofs et al., 2002), utilize *Z*/*E*11-14:OAc mixtures (Ishikawa et al., 1999a) and, thus, there presumably exists the genetic capacity in ACB to express the ECB receptors. The finding of rare males in both ECB and ACB also affords the opportunity to explore the peripheral processing of these compounds in rare and normal individuals in both species (Domingue et al., 2006).

Functional Role of *Z*9-14:OAc and the Evolution of Pheromone Communication in *Ostrinia*

Similar to ECB, the upwind flight response of significant numbers of male ACB was antagonized by the addition of *Z*9-14:OAc to the ACB blend. Glover et al. (1989) showed that addition of 1% *Z*9-14:OAc reduced the level of upwind flight by BE ECB males from 73% to 18%, which is similar to the values reported here for BE males (77% reduced to 17%, Fig. 5). The addition of 1% *Z*9-14:OAc to the ACB blend also resulted in a reduction of upwind flight levels by ACB from 66% to 30%.

The observed antagonist effect of *Z*9-14:OAc on ACB upwind flight is of interest because of its impact on discussions on the phylogenetic relationships within *Ostrinia*, as proposed by Ishikawa et al. (1999a, b). In a study of the host plants and sex pheromones of

Ostrinia species in Japan, Ishikawa et al. (1999a) showed that all species use the Δ11 pheromone components, similar to those of ECB, except for the ACB populations, which use the Δ12 pheromone components. It was proposed that the ACB populations are derived from one of the closely related *Ostrinia* populations that use the Δ11 components. Support for this hypothesis comes from a study of the reductases of ACB. Evolution of the unusual pheromone components of ACB should require a change in the desaturase and a change in the specificity of the reductase for Δ12 instead of Δ11 compounds. However, a study of the ACB reductases (Zhao et al., 1995) revealed that their Δ12 reductase was able to convert *Z*11-14:Acid to the corresponding alcohol more efficiently than it did for the Δ12-14:Acids (with which it seemed to favor the *E* isomer). This suggests a possible close linkage to a *Z*11 strain of *Ostrinia*.

In the study by Ishikawa et al. (1999a), two phylogenies were proposed. In the first, based on morphological data (Mutuura and Munroe, 1970), the group III trilobed uncus species group is divided into two subgroups: one containing ACB, ECB, and *Ostrinia orientalis*; and a second containing *Ostrinia scapulalis*, *Ostrinia zaguliaevi*, and *Ostrinia zealis*. In the second proposed phylogeny, based on mitochondrial COII gene sequence analysis (Ishikawa et al., 1999b; Kim et al., 1999), the subgroups of group III are not supported, and ACB is placed basal to the remaining five species. Based on the existing knowledge about the pheromone systems in *Ostrinia*, the following sequence of changes were proposed to have occurred: (1) the most ancestral form (as yet unidentified) most likely utilized *E*11-14:OAc alone, based on the fact that one of the most basal species, *Ostrinia latipennis*, uses only *E*11-14:OH; (2) species evolved that used mixtures of *Z*/*E*11-14:OAc, such as *Ostrinia palustralis*; (3) species evolved using *Z*/*E*11-14:OAc + *Z*9-14:OAc as a three-component blend (*O. zaguliaevi* and *O. zealis*); (4) species evolved, such as ECB and *O. scapulalis*, in which *Z*9-14:OAc has switched functionality and is an antagonist component of the communication system. Within this scheme, the ACB is hypothesized to be ancestral to the group that includes *O. zaguliaevi* and *O. zealis*, that is, before the incorporation of *Z*9-14:OAc into the pheromone as an agonist component, and in agreement with the second phylogeny based on COII gene sequence analysis. However, if this scheme is correct, then our result, showing that *Z*9-14:OAc also is an antagonist of upwind flight in ACB, implies that the role of *Z*9-14:OAc arose earlier than previously thought, and further that the functionality of this component has switched more than once. Alternatively, our result suggests that species using the three-component pheromone constitute a group that is ancestral to ACB, ECB, and *O. scapulalis* in which *Z*9-14:OAc is an antagonist. This hypothesis is more in agreement with the phylogenetic relationships based on morphological data (Mutuura and Munroe, 1970; Ishikawa et al., 1999a), and the idea that agonist functionality precedes the emergence of antagonism in the evolution of pheromone systems (Linn and Roelofs, 1995; Baker, 2002).

Finally, the addition of the ECB blends did not affect the upwind flight of ACB males to the ACB blend. This result supports the hypothesis that the lack of response of ACB males to the ECB pheromone in experiment 3 was not because of antagonistic properties of the ECB pheromone components, but rather threshold differences between rare ECB-responding ACB males compared with nonresponding ACB males to the ECB blends.

Response Specificity and Additional Pheromone Components for ACB

Male ACB displayed a relatively low level of specificity with respect to blend quality, compared with ECB, evidenced by the similar levels of upwind flight and source contact

(45% to 66%) that occurred with a range of ratios (4:1 to 1:4) of *Z*/*E*12-14:OAc, and the variability in response over the course of the experiments. Previous studies with ECB (Figs. 1b and 2b; Linn et al., 1997) showed that UZ males exhibited a greater specificity for blend ratio than did BE males, with no UZ males flying upwind to a 50:50 ratio. BE males responded to a wider range of ratios (1:99 to 35:65 *Z*/*E*11-14:OAc). With respect to odor quantity, lower specificity was also evidenced by the fact that the maximal level of upwind flights by ACB males was 66%, a value that is considerably less than routinely observed with the UZ ECB males (>90%; see Linn et al., 1997, 2003), but comparable to maximal levels observed with the BE ECB race to their 1:99 *Z*/*E*11-14:OAc mixture (65% to 70%).

Results of other studies with ACB suggest that throughout Asia a range of *Z*/*E*12-14:OAc mixtures are produced by females and result in trap captures of males (Ando et al., 1980; Klun et al., 1980; Cheng et al., 1981; Yeh et al., 1989; Boo and Park, 1998). Field trapping studies and analysis of female pheromone glands at sites in Japan led Huang et al. (2002) to hypothesize that there was a pheromone polymorphism, with populations in the north preferring a blend containing 36% to 39% *E*12-14:OAc, and in the south 44% *E*12-14:OAc. Analysis of field trapping studies in South Korea showed that high numbers of moths were captured with ratios from 3:1 to 1:3 *E*/*Z*12-14:OAc (Boo and Park, 1998).

One explanation for the relatively lower response levels and lack of specificity in ACB is that because the pheromone is made up of unusual Δ14 compounds, there is less pressure from interspecies encounters that could lead to mating mistakes, as might occur in other *Ostrinia* that utilize Δ11 compounds. It also could be the case that other pheromone components are involved in the communication system. Boo and Park (1998) reported the presence of 14:OAc in hexane extracts of female abdominal tips from 2- to 3-d-old females that contained a mean ratio of 0.74:1:1.36 for 14:OAc, *E*12 and *Z*12-14:OAc, but the compound did not elicit an EAG response and was not used in field trials. The field trials showed that among 12 blends tested, the greatest capture occurred with the 2:1 *Z*/*E* mix, but as noted above there was significant variability among sites and in the range of blends capturing high numbers of moths. At one site, a ginger field, maximal captures were reported with a 5:1 *E*/*Z* mixture. Kou et al. (1992) also reported the presence of 14:OAc in the pheromone gland analysis of moths in Taiwan. However, in field trials there was no difference between the three- and two-component mixtures, or a 3:2 *Z*/*E*12 formulation identified in Japan (Ando et al., 1980). Finally, 14:OAc was reported in pheromone gland tips of females in China (Cheng et al., 1981), but when added to the 1:1 *Z*/*E*12 mix at the reported gland content (1.8×sum of isomers), it resulted in reduced trap capture. Our results also suggest that lower levels of upwind flight were not because of a lack of 14:OAc as a potential pheromone component.

Acknowledgements We thank Kathy Poole for help in maintaining the ACB and ECB colonies, and Satoshi Nojima and Shannon Olsson for preparing the solutions for flight tunnel tests. The project was funded by NSF IBN #034340.

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J Chem Ecol (2007) 33:213–217
DOI 10.1007/s10886-006-9212-y

RAPID COMMUNICATION

Chemical Deterrence of a Cyanobacterial Metabolite against Generalized and Specialized Grazers

Edwin Cruz-Rivera · Valerie J. Paul

Received: 16 May 2006 / Revised: 10 October 2006 / Accepted: 16 October 2006 /
Published online: 25 October 2006
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Abstract Pitipeptolide A is the major secondary metabolite in a persistent population of the cyanobacterium *Lyngbya majuscula* from Guam. Because this cyanobacterium is of low preference to many sympatric consumers, we tested the hypothesis that this compound acted as a chemical defense. Pitipeptolide A was deterrent to urchins, two species of amphipods, and small herbivorous crabs, whereas it did not deter feeding by the sea hare *Stylocheilus striatus*, which readily consumes cyanobacteria. Although our comparison included various small invertebrates, which are often tolerant to algal chemical defenses, this cyanobacterial compound proved deterrent at natural concentrations to all but one of the consumer species tested. Thus, the production of defensive secondary metabolites is likely related to the common occurrence of *L. majuscula* on this coral reef despite the abundance of diverse grazers.

Keywords Chemical defense · Cyanobacteria · Guam · *Lyngbya majuscula* · Mesograzers · Pitipeptolide

Introduction

Marine benthic filamentous cyanobacteria have provided natural products chemists with a wealth of novel compounds. This is particularly true for cyanobacteria of the genus *Lyngbya* (Paul et al., 2001). *In vitro* activities of many of these compounds have been studied as part of screening programs in drug discovery, and a number of potent toxins are known. Yet, out of hundreds of compounds isolated and purified, few studies have addressed the ecological roles (Paul et al., 2001; Cruz-Rivera and Paul, 2002).

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Because these benthic cyanobacteria can form extensive blooms that can have detrimental effects on marine life, fisheries and (sometimes) human health, the study of bottom–up *versus* top–down controls of these blooms is important (e.g., Thacker et al., 2001). Herbivory is a strong top–down ecological force controlling the structure and dynamics of marine macroalgae and has favored the evolution of a variety of chemical defenses in seaweeds (Hay and Steinberg, 1992; Paul et al., 2001). Some evidence suggest that chemical defenses could also mediate the persistence of benthic cyanobacteria in areas of high herbivory such as coral reefs (Paul et al., 2001; Cruz-Rivera and Paul, 2002). However, tests of individual compounds against diverse grazers are scarce. This is important because studies testing crude extracts alone (e.g., Cruz-Rivera and Paul, 2002) cannot discern whether the observed deterrence is the result of individual metabolites, or of additive or synergistic interactions among different chemicals. Also, since intraspecific variation in the production of secondary metabolites is well documented for cyanobacteria (Paul et al., 2001), testing individual compounds might provide information on redundancy of roles for diverse compounds endemic to specific populations of the same cyanobacterial species.

In this study, we test a natural role of the major secondary metabolite from a persistent population of the cyanobacterium *Lyngbya majuscula* as a feeding deterrent to sympatric consumers from Guam. We hypothesize that chemical defenses allow mats of this cyanobacterium to persist in an area of high herbivore density and diversity.

Methods and Materials

Lyngbya majuscula was collected from Piti Bomb Holes in Guam, Mariana Islands (13°30′ N, 144°45′E). Large mats of the cyanobacterium were collected by snorkeling, brought to the laboratory, freeze-dried, and extracted in 1:1 ethyl acetate/methanol. After extracting ×3, crude extract was concentrated by using rotary evaporation and partitioned between ethyl acetate and water. The concentrated organic phase was separated by Si Bond-Elut column, and pitipeptolide A eluted with hexane/ethyl acetate (1:3). The mixture was dried and further separated by semipreparative normal-phase HPLC (Econosil Silica) by using an isocratic system of hexanes/ethyl acetate (3:7) (Luesch et al., 2001).

Artificial diets were made following the methods described in Hay et al. (1998), with 4 g of freeze-dried, ground *Ulva* (=*Enteromorpha*) *clathrata*, 0.72 g of agar, and 36 ml deionized water per batch of food. *Ulva* spp. (a number of which were formerly classified under the genus *Enteromorpha*) are routinely used in marine chemical ecology for testing the palatability of compounds because of their lack of chemical or structural defenses that might interact with tested metabolites (Hay et al., 1998). The species used here is readily consumed by a variety of grazers from Guam (Cruz-Rivera and Paul, 2002). Treatment foods were made by coating *U. clathrata* with pitipeptolide A at the natural concentration of 0.8% of dry mass (Pangilinan, 2000). This was accomplished by dissolving the compound in enough ethyl ether to thoroughly soak the powdered alga and then removing the solvent with rotary evaporation. Control foods were made with *U. clathrata* treated with ethyl ether alone. Control or treatment *Ulva* were homogeneously mixed with hot agar and poured individually onto plastic screen mesh to produce food strips. Upon cooling, food-covered screen mesh was cut to desired sizes. For small consumers, the foods were lightly pressed onto strips of screen mesh. For urchins, which consume larger amounts, foods were poured onto molds that were pressed against plastic screen (Hay et al., 1998). Consumption was quantified as the number of squares cleared of food by the grazers.

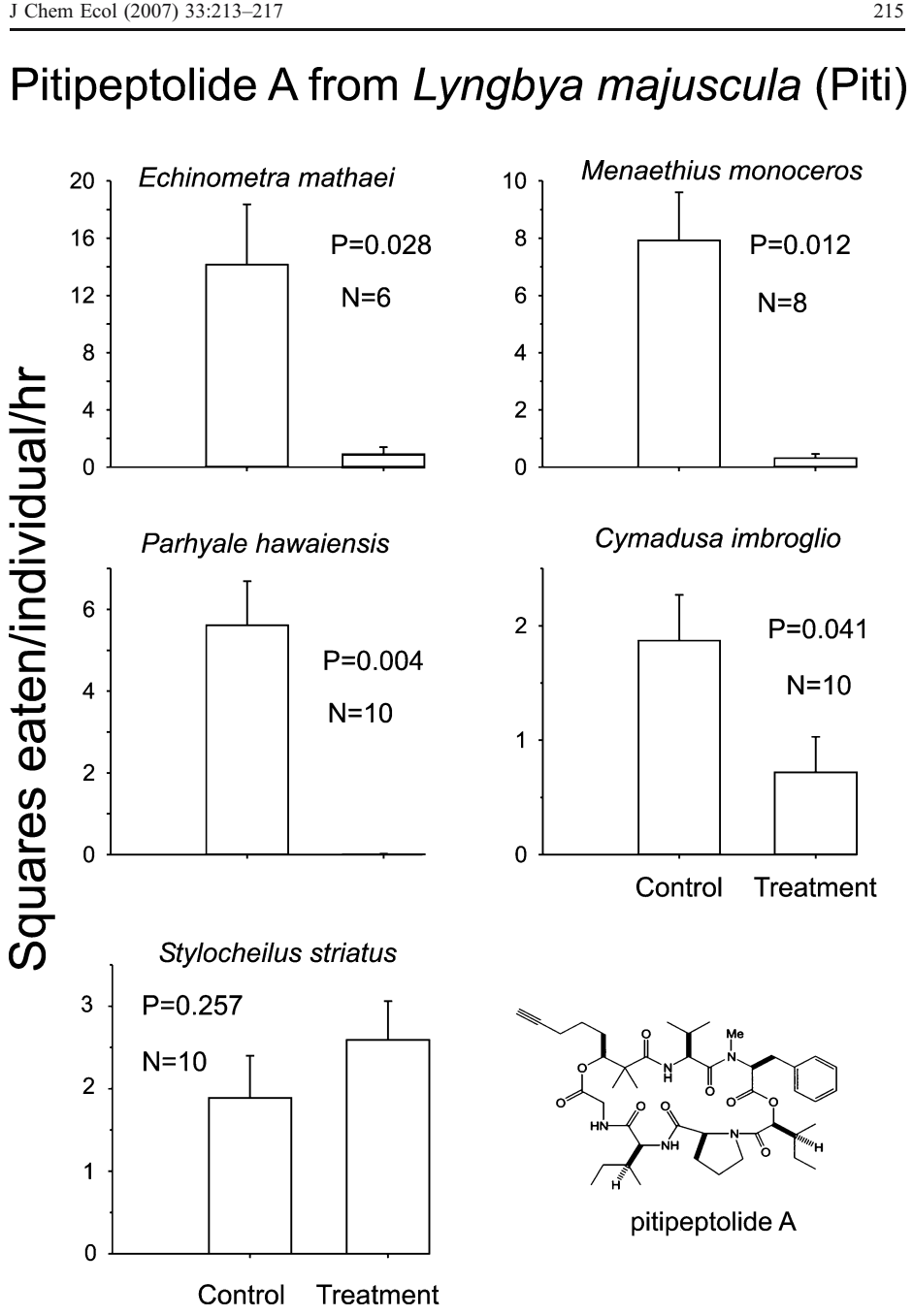


Fig. 1 Consumption of artificial diets containing or lacking pitipeptolide A by five invertebrate consumers from Guam. Bars represent mean (±1 SE) amount of food eaten by individuals per hour (see Methods and Materials). Analyses are by paired *t*-tests

Treatment and control foods were simultaneously offered to five invertebrate consumers: the sea urchin *Echinometra mathaei*, the small majid crab *Menaethiusmonoceros*, the gammaridean amphipods *Parhyalehawaiensis* and *Cymadusa imbroglio*, and the small sea hare *Stylocheilus striatus* (= *S. longicauda*; see Rudman, 1999). Grazers were obtained from algal and cyanobacterial mats collected by snorkeling at Piti Bomb Holes, Pago Bay, and from flow-through tanks at the University of Guam Marine Laboratory. The tanks receive unfiltered water from the adjacent reef, and organisms continuously recruit from the field.

Experiments with urchins were conducted in flow-through 20-l tanks (N=10). For all other smaller consumers, 16 cm (diam)×6 cm (height) dishes were used (N=10). Replicates contained single individuals of each consumer species except for the amphipods. Because *P. hawaiensis* would invariably form precopulatory pairs, and mate guarding could influence feeding behavior, we used four males or four females per replicate. For *C. imbroglio*, the smallest of the consumers tested, five individuals were placed in each replicate. Experiments were monitored over 24 hr, and replicates were scored when more than 50% of either treatment or control foods were eaten. Paired t-tests were used to analyze data (squares eaten/individual/hr). Replicates in which no food was consumed after 24 hr were eliminated from the analyses as they offered no information of feeding preference.

Results

Pitipeptolide A at natural concentrations deterred feeding in all but one of the invertebrates grazers tested. The urchin *E. mathaei*, the small algal-dwelling crab *M. monoceros*, and the amphipods *P. hawaiensis* and *C. imbroglio* all consumed significantly more of the control algal food (without pitipeptolide A) than of the treatment food (P=0.004–0.41, Fig. 1). The exception to this pattern was the small sea hare, *S. striatus*, which was not deterred by natural concentrations of the compound (P=0.257, Fig. 1).

Discussion

While hundreds of compounds have been isolated from marine benthic cyanobacteria, and *L. majuscula* in particular, only a handful of studies have addressed the ecological roles of cyanobacterial secondary metabolites (reviewed in Paul et al., 2001). In this work we demonstrate a defensive role for the main secondary metabolite produced by a specific *L. majuscula* population from Guam. Pitipeptolide A was deterrent at natural concentrations against urchins as well as three smaller algal/cyanobacterial-dwelling grazers. Previous studies have shown that *L. majuscula* from this population is also a low preference food for sympatric fishes, suggesting that its chemical defenses might act against a broad array of consumers (Cruz-Rivera and Paul, 2002).

The exception to this pattern of deterrence was the small gastropod *S. striatus*. This sea hare has been collected from at least six cyanobacterial genera and will readily consume cyanobacteria (Cruz-Rivera and Paul, 2002, 2006). It will also accumulate compounds from *L. majuscule*, and their role as potential sequestered chemical defenses has been discussed (Paul et al., 2001; Capper et al., 2005). While it could be expected that this grazer would not be deterred by *Lyngbya* extracts or compounds, studies have shown that different *L. majuscula* compounds and their varying concentrations may stimulate or deter feeding by *S. striatus*, which does not appear to be completely adapted to *L. majuscula* secondary

metabolites (Paul et al., 2001; Cruz-Rivera and Paul, 2002). In this study, however, the natural concentration of pitipeptolide A did not deter feeding by this sea hare.

Other consumers tested here are more generalized than *S. striatus* and will eat macroalgae, sometimes as much as cyanobacteria (Cruz-Rivera and Paul, 2002, 2006). Mesograzers such as the amphipods and crabs used here are often better adapted to secondary metabolites of macroalgae. Living on chemically defended hosts can reduce predation from fishes, although closely related mesograzers can vary greatly in their ability to use a chemically defended host (Hay and Steinberg, 1992; Paul et al., 2001). It is possible that similar advantages are experienced by small consumers preferring, or specializing on, chemically defended cyanobacteria as *S. striatus* does (Cruz-Rivera and Paul, 2002, 2006). If so, then tropical benthic cyanobacteria could fill similar niches as chemically defended macroalgae (as food and shelter) for small marine consumers that have adapted to live, and feed, on benthic cyanobacterial mats (Cruz-Rivera and Paul, 2002).

Acknowledgments Funding for this study was provided by an NIH Postdoctoral Supplement (to E. Cruz-Rivera) to NIH grant CA 53001 (J. Horwitz, P.I.). We thank the reviewers whose comments improved this manuscript. This is contribution # 589 of the University of Guam Marine Laboratory and contribution # 670 from the Smithsonian Marine Station at Fort Pierce.

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J Chem Ecol (2007) 33:219–223
DOI 10.1007/s10886-006-9225-6

RAPID COMMUNICATION

Lichen Substances Affect Metal Adsorption in *Hypogymnia physodes*

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Received: 1 October 2006 / Accepted: 13 November 2006 /
Published online: 29 November 2006
© Springer Science + Business Media, Inc. 2006

Abstract Lichen substances are known to function as chetators of cations. We tested the hypothesis that lichen substances can control the uptake of toxic metals by adsorbing metal ions at cation exchange sites on cell walls. If true, this hypothesis would help to provide a mechanistic explanation for results of a recent study showing increased production of physodalic acid by thalli of the lichen *Hypogymnia physodes* transplanted to sites with heavy metal pollution. We treated cellulose filters known to mimic the cation exchange abilities of lichen thalli with four lichen substances produced by *H. physodes* (physodic acid, physodalic acid, protocetraric acid, and atranorin). Treated filters were exposed to solutions containing seven cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Na^{+}), and changes to the solution concentrations were measured. Physodalic acid was most effective at influencing metal adsorption, as it increased the adsorption of Fe^{3+}, but reduced the adsorption of Cu^{2+}, Mn^{2+}, and Na^{+}, and to a lesser extent, that of Ca^{2+} and Mg^{2+}. Reduced Na^{+} adsorption matches with the known tolerance of this species to NaCl. The results may indicate a possible general role of lichen substances in metal homeostasis and pollution tolerance.

Keywords Depsides · Depsidones · Pollution tolerance · Epiphytic lichens · Transition metals · Alkaline earth metals · Alkali metals

Introduction

Lichens are notable for the production of unique lichen substances, including more than 800 aliphatic, cycloaliphatic, aromatic, and terpenic compounds (Huneck and Yoshimura, 1996). Lichens are also sensitive to a variety of air pollutants and are widely used as

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biomonitors of air quality. Recently, Białońska and Dayan (2005) linked lichen secondary chemistry with the use of lichens as biomonitors in a study of the common lichen *Hypogymnia physodes*. This lichen has often been employed as a model species in ecophysiological and biomonitoring studies because of its abundance and its moderate sensitivity to SO_2 and heavy metals. In Europe, *H. physodes* has declined in many areas with significant pollutant load. Białońska and Dayan (2005) found that thalli of *H. physodes* transplanted to areas polluted with heavy metals and inorganic S compounds exhibited significantly lower concentrations of atranorin, physodic acid, and 3-hydroxyphysodic acid and significantly higher concentrations of physodalic acid. They postulated that physodalic acid might participate somehow in the stress response of *H. physodes* to toxic effects of heavy metals and acidic inorganic S compounds. This was the first time that the content of lichen substances had been linked to pollution tolerance. However, the authors did not provide any mechanistic hypothesis to explain any such relationship.

Our hypothesis is that the presence of lichen compounds significantly reduces toxic effects of heavy metals to living cells by controlling their extracellular adsorption and thereby influencing their intracellular uptake. Specifically, we tested the hypothesis that physodalic acid affects the adsorption of metal ions at cation exchange sites and thereby contributes to the heavy metal tolerance of *H. physodes*. To test our hypothesis, we used cellulose filters coated with physodalic acid and other lichen substances of *H. physodes* (atranorin, physodic acid, and protocetraric acid) as a standardized model system containing numerous hydroxyl groups as cation exchange sites (Hauck et al., 2007). Lichen cell walls and exopolysaccharide matrices harbor many hydroxylic and carboxylic binding sites for cations, from which delayed intracellular uptake is possible (Hauck et al., 2006). Metal ions chosen for the study included the transition metals Cu^{2+}, Fe^{2+}, Fe^{3+}, and Mn^{2+} and the alkaline earth metals Ca^{2+} and Mg^{2+}, which (1) occurred in measurable concentrations in the stemflow of trees with *H. physodes* in ecosystem studies conducted in Europe and North America (Hauck, 2003); (2) are essential macro- or micronutrients; and (3) are, in the case of the transition metals, toxic to *H. physodes* when concentrations significantly exceed the amount required for nutrition (Hauck, 2003). The alkali metal Na^{+} was studied in addition, as it is often the most common metal in precipitation.

If our hypothesis is correct, the presence of lichen compounds on cellulose filters will significantly alter the adsorption of metal cations from solution. We were especially interested in the effects of physodalic acid on cation uptake inasmuch as this compound was specifically mentioned by Białońska and Dayan (2005) to be involved in the stress response of *H. physodes*.

Methods and Materials

Hypogymnia physodes (L.) Nyl. is a common foliose lichen of the temperate and boreal zones of the northern hemisphere, which primarily occurs as an epiphyte (McCune, 2002). Seven lichen substances have been identified in *H. physodes* (McCune, 2002), which are either located in the cortex (atranorin, chloroatranorin) or in the medulla (physodic, 3-hydroxyphysodic, physodalic, protocetraric, 2′-*O*-methylphysodic acids). The two latter substances are found only in minor amounts (McCune, 2002). The cortical lichen substances of *H. physodes* are depsides, whereas the medullary ones belong to the depsidones (Huneck and Yoshimura, 1996).

Pure atranorin, physodic acid, physodalic acid, and protocetraric acid from the collection of S. Huneck were dissolved in acetone (2.5 mM) and applied to ash-free cellulose filter

Table 1 Cation adsorption from micromolar salt solutions by filter paper soaked with 2.5 mM of different lichen substances

	Cu^{2+}	Fe^{2+}	Fe^{3+}	Mn^{2+}	Ca^{2+}	Mg^{2+}	Na^{+}
Incubation medium	8	85	85	35	260	110	800
Physodalic acid	1.21±0.27 (119%[a])[b]	65.6±2.6 (−3%)	1.2±1.1 (−95%)[b]	23.7±0.4 (23%)[b]	242±3 (4%)[b]	90.9±2.2 (5%)[b]	837±35 (48%)[b]
Physodic acid	0.71±0.16 (28%)	68.6±2.2 (1%)	14.0±3.1 (−44%)[b]	19.1±0.2 (−1%)	238±2 (3%)	84.2±1.3 (−3%)	759±31 (34%)[b]
Protocetraric acid	1.02±0.12 (84%)	69.8±1.4 (3%)	14.4±2.1 (−43%)[b]	22.8±0.5 (18%)[b]	238±3 (2%)	89.6±2.1 (3%)	740±37 (31%)[b]
Atranorin	0.76±0.15 (37%)	63.7±2.1 (−6%)	27.3±3.6 (8%)	20.3±0.4 (9%)	245±5 (5%)[b]	88.7±1.9 (2%)	782±26 (38%)[b]
Control	0.55±0.12	67.7±1.2	25.2±3.1	19.3±0.3	233±1	86.8±2.5	565±64

Micromolar salt solutions are chloride salts, except for Na_2SO_3. All concentrations in μM. Arithmetic means ± standard error, $N=5$.

[a] Relative change of metal ion concentration after shaking two filter strips impregnated with different lichen substances or not (control) for 1 hr in salt solution. Control is set to 100%. Positive values indicate higher and negative values indicate lower metal concentration in the solution.

[b] Significant difference from control (Duncan's multiple range test, $P \leq 0.05$, $df=20$).

paper (Blue Ribbon Filters, Schleicher & Schuell, Dassel, Germany) by shaking filter strips (ca. 20 cm^2, 160 mg) in the lichen substance solution for 1 hr. Cellulose filters consist of randomly interlaced fibers that are littered with exchange sites that bind to metal ions, similar to the cell wall surfaces of lichens (Hauck et al., 2007). Untreated filter strips were used as controls. Two strips of impregnated filters per replicate were exposed for 1 hr to 25 ml of micromolar solutions of $CuCl_2$, $FeCl_2$, $FeCl_3$, $MnCl_2$, $CaCl_2$, $MgCl_2$, or Na_2SO_3; the concentrations applied (Table 1) referred to the optimum measuring range of the AAS (Vario 6, Analytik Jena, Jena, Germany; Cu, Fe, Mn, Ca, Mg) or inductively coupled plasma-atomic emission spectrometry (Spectraflame, Spectro Analytical Instruments, Fitchburg, MA, USA; Na) used for chemical analysis. After incubation, the filter paper was removed with forceps and metal concentrations in the solution were analyzed. The experiment was run in five replicates. Statistical analyses were conducted with SAS 6.04 software (SAS Institute, Cary, NC, USA). Duncan's multiple range test was applied to test for significance of differences between means after testing for normal distribution with the Shapiro–Wilk test.

Results

For all but one of the cations studied, the presence of lichen compounds on filter strips resulted in significantly reduced removal of the cations from solution, indicating that lichen compounds prevent the binding of cations to exchange sites on the filters. Physodalic acid was especially inhibitory inasmuch as filters coated with this lichen compound adsorbed from solution significantly less Cu^{2+}, Mn^{2+}, Na^{+}, Ca^{2+}, and Mg^{2+} than control samples of filters not soaked with a lichen substance (Table 1). Adsorption of Mn^{2+} was also reduced by protocetraric acid, as was that of Ca^{2+} by atranorin. All studied lichen substances also inhibited the adsorption of Na^{+}. Lichen compounds caused significantly enhanced adsorption of only one metal cation, and this was Fe^{3+}. While the concentrations of Fe^{2+}

were not affected by any of the lichen substances, Fe^{3+} was nearly completely removed from solution by filters coated with physodalic acid. Physodic and protocetraric acids significantly increased removal of Fe^{3+} from the incubation medium by 43–44% (Table 1).

Discussion

Our results provide a mechanistic explanation for the hypothesis of Białońska and Dayan (2005) that enhanced synthesis of physodalic acid in polluted areas is a stress response of *H. physodes* to heavy metal pollution. High ambient concentrations of both Cu^{2+} and Mn^{2+} have been shown to limit the abundance of *H. physodes* in the field (Hauck, 2003; Hauck and Paul, 2005). An inhibition of the adsorption of these ions in the apoplast would, therefore, increase the tolerance of *H. physodes* to Cu^{2+} and Mn^{2+}.

The strong reduction of the Fe^{3+} concentration in the incubation medium by physodalic acid suggests that this lichen substance forms complexes with Fe^{3+}. Evidence for the existence of such complexes with Fe^{3+} has been found for several lichen substances (Engstrom et al., 1980; Hauck et al., 2007) belonging, like physodic acid, to the depsidones (fumarprotocetraric and norstictic acids), the depsides (divaricatic acid), the pulvinic acid derivatives (pulvinic and rhizocarpic acids), or the anthraquinones (parietin). Complex formation with transition metals has also been established for other combinations of lichen substances and metal ions (e.g., Cu^{2+}) and was mostly discussed in the light of immobilization of potentially toxic ions (Takani et al., 2002).

Occurrence of Fe^{3+}-adsorbing lichen substances in lichens of sites with low Fe availability, but lack in most lichen species of Fe-rich substrate, suggests that Fe^{3+} bound in complexes with lichen substances is subsequently taken up into the cytoplasm (Hauck et al., 2007). The only Fe^{3+} adsorbing lichen substances known, so far, from lichen species of Fe-rich substrate, viz. norstictic and rhizocarpic acids, simultaneously reduce the adsorption of Fe^{2+} (Hauck et al., 2007). Increased uptake of Fe^{3+} in *H. physodes* by physodalic acid and other lichen substances would increase its tolerance to Mn^{2+}, as Mn-related damage depends on the Mn/Fe ratio (Hauck and Paul, 2005).

If our hypothesis is correct, the increased production of physodalic acid by *H. physodes* in polluted areas observed by Białońska and Dayan (2005) is a response to pollution stress. However, the reduced concentration of other lichen substances (atranorin, physodic acid, and 3-hydroxyphysodic acid) also requires explanation. The decline of physodic acid in thalli of *H. physodes* transplanted to sites with heavy metal pollution could be due to conversion into the chemically related physodalic acid. Protocetraric acid, which is even more closely related to physodalic acid than physodic acid (Huneck and Yoshimura, 1996), was not included in the analyses of Białońska and Dayan (2005) and should be investigated for possible stress responses. Inhibition of Na^{+} adsorption by all investigated lichen substances (Table 1) agrees with the known tolerance of *H. physodes* to NaCl (Punz, 1979).

The putative ability of physodalic acid to increase heavy metal tolerance would explain why *H. physodes* was less subject to pollutant-caused declines in the past than other European *Hypogymnia* species, including *Hypogymnia farinacea*, *Hypogymnia tubulosa*, and *Hypogymnia vittata*, as *H. physodes* is the only European species with physodalic acid (McCune, 2002). The assumed protective effect of physodalic acid in *H. physodes* is probably limited because this substance is primarily produced by the fungal hyphae of the medulla and the algal layer (McCune, 2002), while the cortical atranorin did not affect the adsorption of transition metals (Table 1). The absence of a protective lichen substance from the cortex would explain the moderate heavy metal tolerance of *H. physodes* (Hauck, 2003;

Hauck and Paul, 2005). *Lecanora conizaeoides*, a markedly Mn-tolerant lichen (Hauck and Paul, 2005), contains the depsidone fumarprotocetraric acid in the entire thallus. This substance also reduces Mn^{2+} adsorption and is even more effective at adsorbing Fe^{3+} than physodalic acid (Hauck et al., 2007). Though our experiment suggests a protective role of physodalic acid, it has to be kept in mind that adsorption rates on fungal and algal cell walls might differ from that of cellulose fibers. Moreover, other mechanisms including immobilization in polyphosphate bodies and phytochelatines are involved in the heavy metal tolerance of lichens (Hauck and Paul, 2005).

Acknowledgments The study was supported by a grant of the Deutsche Forschungsgemeinschaft to M. Hauck (Ha 3152/8-1). Inductively coupled plasma analyses were carried out in the Institute of Soil Science and Forest Nutrition, University of Göttingen (Dr. Norbert Lamersdorf). Dr. James D. Lawrey, George Mason University, Fairfax, VA, is warmly thanked for extensive comments on the manuscript and for checking the English language.

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J Chem Ecol (2007) 33:225–237
DOI 10.1007/s10886-006-9226-5

Zea Mays: Benzoxazolinone Detoxification Under Sulfur Deficiency Conditions—A Complex Allelopathic Alliance Including Endophytic *Fusarium Verticillioides*

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Received: 3 August 2006 / Accepted: 16 November 2006 /
Published online: 3 January 2007
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Abstract Benzoxazolinone detoxification is similar in plants grown under sulfur deficiency conditions and in control plants grown with an optimal S supply. However, when S-deficient plants were treated with metolachlor before benzoxazolin-2(3H)-one (BOA) incubation, detoxification was reduced, as indicated by a lower accumulation of the detoxification products BOA-6-*O*-glucoside and glucoside carbamate and by a loss of BOA-6-OH glucosyltransfease activity. Root colonizing microorganisms and the endophytic fungus *Fusarium verticillioides* participated in benzoxazolinone detoxification by converting the compound to 2-acetamidophenol (AAP) or *O*-hydroxyphenyl malonamic acid (OHPMA), a process accompanied by the appearance of phenoxazinone. Maize roots, however, absorbed AAP and OHPMA only in traces. Absorbed traces of OHPMA stimulated maize radicle growth, and traces of AAP stimulated cress. Phenoxazinone inhibited the growth of cress radicles at

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concentrations higher than 500 μM, whereas maize radicles were hardly affected. *F. verticillioides* did not convert benzoxazolinone to any known microbial degradation product when the medium of maize seedlings was inoculated with the fungus under sterile condition. Plant–fungus interactions seem to be important in plant survival of allelopathic attacks. This study points to a complicated network of allelopathic interactions that are influenced by biotic and abiotic factors, including herbicides.

Keywords Allelopathy · Benzoxazolinone · Detoxification · *Fusarium verticillioides* · Phenoxazinone · Sulfur deficiency · *Zea mays*

Introduction

Allelopathic interactions between plants are known to be complex depending on both biotic and abiotic factors that influence them. Soil microorganisms are an important component of allelopathic interactions, underlying environmental influences that change seasonally. Studies of allelopathy under field conditions, therefore, often involve uncontrolled variables and often produce unpredictable or irreproducible results, leading to misinterpretations. The use of model systems reduces the complexity and allows a more precise and defined characterization of molecular events and responses of a plant on treatments with allelochemicals. Current opinion in allelopathy holds that the inhibitory or stimulating effects of allelochemicals become more evident when combined with additional biotic and abiotic stress situations experienced by plants during development (Einhellig 2003).

Benzoxazinones, secondary products of several monocots such as rye, wheat, and maize and of some dicots such as several *Lamium* species, are biologically active compounds that are relatively phytotoxic (Gierl and Frey, 2001; Sicker and Schulz, 2002; Alipieva et al., 2003; Macias et al., 2005). As aglucones, they become unstable and are converted to benzoxazolin-2 (3H)-ones (BOAs), which have a reduced phytotoxicity in comparison to the original compounds. The benzoxazolin-2(3H)one, for instance, is a known allelopathic compound thought to be mainly responsible for the phytotoxic properties of rye mulch that suppresses several weeds common in North America (Barnes et al., 1986). The mechanism of action of the benzoxazolinones as phytotoxins is unknown, but effects have been found on *in vivo* protein and nucleic acid synthesis. However, some plants are able to overcome the negative effects because they have developed mechanisms to detoxify the compounds. In *Arabidopsis thaliana*, for instance, 158 genes are induced by BOA, many of them potentially involved in detoxification processes (Baerson et al., 2005).

Benzoxazolin-2(*3H*)-one has been used in model systems to elucidate the detoxification capacity of monocotyledonous and dicotyledonous weeds and crops found in European field communities (Schulz and Wieland, 1999, Sicker et al., 2000). Existing variations in the detoxification efficiencies are thought to be jointly responsible for the differences often observed among dicots and monocots in their sensitivity to BOA. The most important detoxification product accumulating temporarily in cereals and several other Poaceae after BOA incubations was identified as glucoside carbamate (Sicker et al., 2001; Wieland et al., 1999) (Fig. 1). This product, not toxic to higher plants such as cress up to 1 mM concentrations, can be subsequently modified in maize to the corresponding gentiobioside or to 1-(2-hydroxyphenylamino)-6-*O*-malonyl-1-deoxy-β-glucoside 1,2-carbamate (malonyl glucoside carbamate (Hofmann et al., 2006). A second important metabolite has been identified as BOA-6-*O*-glucoside (Wieland et al., 1998; Sicker et al., 2003; Schulz et al., 2006). This compound is the major detoxification product found in most of the dicots, where glucoside

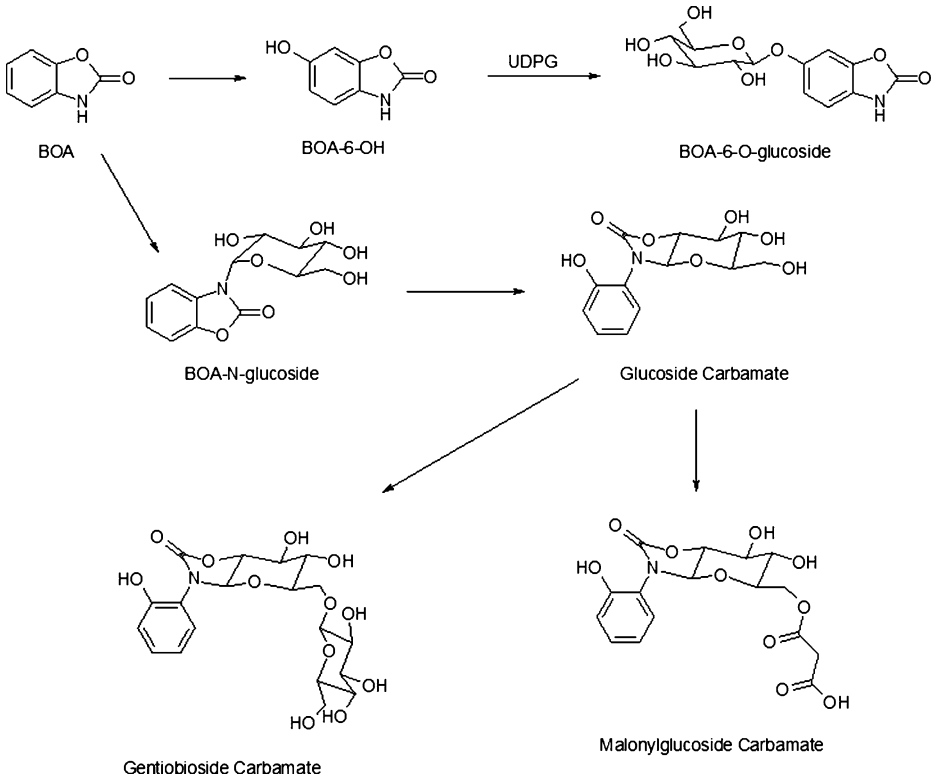


Fig. 1 Detoxification pathways in maize roots

carbamate was produced only in traces or not at all. Accumulation of extractable detoxification products was transient in maize. At least a portion of them was exuded by the roots following partial or complete BOA absorption or after a change to BOA-free medium. The roots are the most important plant organ for BOA detoxification, and shoots are only involved when roots are overloaded. Among the 30 species tested for BOA detoxification capacity, *Zea mays* was the most effective (Schulz and Wieland, 1999). Application of 125 μmol BOA to 30 6-day-old seedlings never led to an accumulation of any detoxification products in the shoot.

Maize was chosen to address the question whether BOA detoxification is affected when additional stress conditions act on the plant. With regard to induced or up-regulated enzymes important for detoxification reactions, such as glucosylation, protein synthesis should be a critical process in the efficiency of detoxification. As sulfur deficiency affects protein synthesis, it was chosen as a stress condition that might influence BOA detoxification. As an additional stress factor, treatment with the synthetic herbicide metolachlor was performed.

Methods and Materials

Plant Cultivation Maize (*Zea mays* L. cv. Nicco) seeds were planted 2 cm deep in pots (diameter 17 cm) filled with perlite (Perligran G; Germany; granulometric composition: 0–3 mm). Seven days after sowing, extra plants were removed to leave four uniform seedlings

per pot. Plants were grown in a greenhouse at 20°C during the light period and a minimum temperature of 16°C at night. Pots were watered daily with diluted nutrient solution. Over a period of 2 wk, the following amounts of fertilizers were applied: 1,660 mg K (K_3PO_4, K_2SO_4, or K_2HPO_4), 654 mg P (K_3PO_4, Na_2HPO_4, NaH_2PO_4, or K_2HPO_4), 500 mg N (NH_4NO_3), 103 mg Mg ($MgCl_2$), 100 mg Fe ($FeCl_3$), 20 mg Mn ($MnCl_2$), 20 mg Zn ($ZnCl_2$), 20 mg Cu ($CuCl_2$), 5 mg B (H_3BO_3), 5 mg Mo (Na_2MoO_4), 5 mg Co ($Co(NO_3)_2$), and 1,000 mg Ca ($CaCO_3$) per pot. Two different S-levels according to the fertilized amount of S per pot were applied: 0 mg S (S_0) and 200 mg S (S_{200}) as SO_4^{2-}. Plants were grown for 3 wk.

In one series of experiments, S_0 and S_{200} plants were treated with metolachlor, (DualGold®; 0.4% v/v spray concentration) 3 wk after sowing. Another application was given 2 d later. Treatment with BOA was started 24 hr after the second metolachlor application.

For incubations, experiments with 2-acetamidophenol (AAP) or *O*-hydroxyphenyl malonamic acid (OHPMA) and for fungus identification, maize seedlings were grown aeroponically on cheesecloth. Seedlings (5 d old) were used for uptake analysis and for cultivation on agar to isolate the fungus.

Sulfur Determination in Seeds After drying seeds of *Zea mays* at 90°C and grinding with an attrition mill, the S contents were analyzed by a C/N/S-Elemental-Analyser (Euro EA).

Incubation and Extraction of Maize Plants with Allelochemicals Plants were carefully removed from the pots and incubated with 500 μM BOA (Sigma) incubation medium as described by Schulz and Wieland (1999) for 24 hr. After incubation, the roots were harvested, mortared with quartz sand, and extracted with 50% methanol. Homogenates were centrifuged at 20,000×*g* for 15 min and at 4°C. Supernatants were used for high performance liquid chromatography (HPLC) analysis. The incubation medium was treated with ethyl acetate, the resulting organic phases and the water phases were evaporated to dryness, and the residues were dissolved in methanol. After centrifugation, the solutions were analyzed by HPLC.

For incubation experiments with 100 μM 2-acetamidophenol (Aldrich) or with 100 μM *O*-hydroxyphenyl malonamic acid for 24 and for 48 hr, respectively, 5-d-old seedlings were incubated. Seedlings were separated into roots and shoots and extracted with methanol as described above. Extracts were analyzed by HPLC.

HPLC analyses were performed with a Beckman Coulter pump module 126 and a diode array detector 166. For separation, an Ultraphere ODS 18 RP column was used. Elution was performed with 0.1% trifluoro acetic acid in H_2O as eluent A and methanol as eluent B with the following gradient: 1 min: 100% A; 1–21 min 20% B in A, linear; 21–41 min 100% B, linear; 43 min 100% B. The detection wavelengths were 280 and 405 nm.

Further allelochemicals used for incubations and as reference substances were BOA-6-OH (Wieland et al., 1999) and *O*-hydroxyphenyl malonamic acid (Friebe et al., 1998). 2-Amino-3*H*-phenoxazin-3-one (phenoxazinone) was synthesized as described in Friebe et al. (1996) but extraction of the methanolic solution was performed with chloroform.

Glucosyltransferase Assay Roots were extracted at 4°C with 100 mM 2-morpholinoethanesulfonic acid monohydrate (MES) pH 6.0 containing 10% glycerol, 1% Na-ascorbate, and 1 mM dithiothreitol (DTT) (1:3 w/v) and 1% protease inhibitor cocktail (Sigma) by mortaring with quartz sand. The homogenate was centrifuged at 20,000×*g* (4°C) for 20 min, the supernatant was used as crude protein extracted for enzyme assays. The assay consisted of 68 μl protein solution, 50 mM Tris, 2.5 mM Uridine diphosphate glucose (UDPG), and

200 μM of synthetic BOA-6-OH (Wieland et al., 1999) in a total volume of 100 μl. After incubation at 30°C for 30 min, the reaction was stopped by boiling for 10 min. The mixture was centrifuged at 20,000×*g* for 5 min and analyzed by HPLC.

Identification of the Endophytic Fungus Maize roots were cut into pieces and transferred to Petri dishes containing Czapek Iprodione Dicloran agar (CZID-agar according to Abildgren et al., 1987) and incubated in the dark at 25°C. Mycelium appearing on agar medium was purified and transferred onto Czapek Dox agar for identification.

All other *Fusarium* isolates utilized in this study were taken from the Fungal Collection (Institute of Microbiology and Virology by D.K. Zabolotny, Ukraine) and maintained on Czapek Dox agar.

For microscopic characterization of *Fusarium* sp. isolated from maize roots, the fungus was cultivated on synthetic nutrients agar (SNA) for 14 d at 25°C under near UV-light to allow spore formation (Nirenberg, 1976). Spores were observed microscopically with a Leitz DMRB microscope equipped with Normanski interference and using the key given by Nelson et al. (1983).

Polymerase chain reaction (PCR) parameters: after 7 d of culture on Czapek Dox agar, the isolated *Fusarium* sp. was used for species-specific identification with molecular techniques. Fungal cells were scraped off agar plates and used for PCR amplification. PCR was accomplished in 25-μl PCR mixtures containing species-specific primers 53-6F/53-6R (Möller et al., 1999) and FOF1/FOR1 (Mishra et al., 2003), Taq DNA Polymerase (1.25 U, Fermentas), KCl-buffer, $MgCl_2$ (25 μM), deoxyribonucleotide triphosphates (dNTPs) (10 mM, Amersham) and performed as follows: one cycle at 95°C for 5 min; 35 cycles at 95°C for 30 sec, 65°C (primer pair 53-6F/53-6R) and 58°C (primer pair FOF1/FOR1) for 30 sec, and 72°C for 2 min 30 sec; followed by a 7-min extension at 72°C; and hold at 4°C. Amplification products were separated on a 1% agarose gel run at 100 V and visualized by ethidium bromide staining of the agarose gel.

Incubation of Fusarium verticillioides with Allelochemicals Mycelial plugs from agar plates (discs 5 mm diameter with 0.5–1 mg mycelium) were transferred into 250-ml flasks with 100 ml sterilized, liquid Czapek medium. The incubations were performed at 27°C in the dark without and with addition either of 2-acetamidophenol, *N*-(2-hydroxyphenyl) malonamic acid (each 10 μmol), or 2-amino-3H-phenoxazin-3-one (1 μmol). The structures of the compounds are presented in Fig. 4.

Cultures were analyzed after 2 wk of cultivation. Mycelia were separated from the liquid by filtration through a 200-μm nylon net and weighed, and the incubation media were extracted with ethyl acetate. The organic and aqueous phases were evaporated to dryness, and the residues were dissolved in 70% methanol. The resulting solutions were analyzed by HPLC.

Radicle Growth Tests Radicle growth tests were conducted in Petri dishes (12 cm diameter), (Schulz and Wieland, 1999). Filter paper in the Petri dishes was saturated with 2 ml methanol or with 2 ml chloroform only, or with 2 ml of solutions of 100, 250, 500, and 1,000 μM *N*-(2-hydroxyphenyl) malonamic acid, 2-acetamidophenol (both dissolved in methanol) and 2-amino-3H-phenoxazin-3-one (dissolved in chloroform). After complete evaporation of the solvent, 15 imbibed maize caryopses were placed on the filter paper and watered with 2 ml tap water. The Petri dishes were sealed with parafilm. The length of the radicle was determined 3 d after germination at 25°C in the dark. Determinations of radicle growth were also

performed with *Lepidium sativum*, cress (30 seeds per Petri dish). Conditions were the same as described with maize. The tests were repeated six times.

Incubation of Sterilized Maize Seedlings Inoculated with F. verticillioides Isolated from Maize Roots and/or Doratomyces stemonitis in the Presence of BOA Sterilized maize caryopses were germinated on Murashige and Skoog (MS) medium in Petri dishes under sterile conditions in a growth chamber at 25°C and a photoperiod of 16 hr. When the seedlings had developed one to two leaves, they were transferred to tubes filled with l0 ml sterilized liquid MS medium containing 100 or 740 μM BOA. The medium was inoculated with *D. stemonitis*, used as a nonpathogenic control, or *F. verticillioides*, respectively, or with both fungi. After 10 days, the liquid was collected and dried by vacuum centrifugation. The residue was dissolved in 50% methanol, the resulting solution was centrifuged and the supernatant used for HPLC analysis.

Results

Plant Detoxification of BOA Three-week-old plants suffering from sulfur deficiency showed reduced growth and reddish leaves due to anthocyanin accumulation, which indicates stress reactions. The appearance of the S_0 plants was an indicator that the low sulfur reserves of the caryopses (0.074–0.076 S%) were depleted during growth and that SO_2 uptake from the air was not sufficient to cover the sulfur requirement. Metolachlor-treated S_0 and S_{200} plants developed necrotic spots on the leaves, but there was no visible additional deterioration when plants were finally incubated with 500 μM BOA for 24 hr.

HPLC analysis of the methanolic root extracts revealed no differences in the content of BOA-6-*O*-glucoside regardless of whether plants were grown under optimal or deficient sulfur supplies (Table 1). Compared to the S_{200} variant, the glucoside carbamate content was slightly higher in roots of sulfur-deficient plants. However, metolachlor treatment strongly affected detoxification, and differences could be observed between metolachlor-treated S_{200} and S_0 plants. In the S_0 variant, the content of BOA-6-*O*-glucoside and glucoside carbamate dropped compared to that of S_0 plants without metolachlor treatment. In S_{200} plants, glucoside carbamate accumulation was slightly diminished, whereas BOA-6-*O*-glucoside production was higher than that found in S_{200} plants without metolachlor treatment.

The accumulation pattern of BOA-6-*O*-glucoside is in accordance with the UPDG-glucosyltransferase activity involved in BOA-6-OH glucosylation. The enzyme responds to BOA and metolachlor treatments. The enzyme activity was apparently affected by metolachlor in both sulfur-deficient and S_{200} plants (Table 2). There was no increase of the enzyme activity when S_{200} plants were treated with metolachlor only. In sulfur-deficient

Table 1 Content of the major BOA detoxification products in maize roots

Detoxification Compound (/g FW)	S(-) Plants	S_{200}-plants	Metolachlor Treated S(-)Plants	Metalochlor Treated S_{200} Plants
BOA-6-*O*-glucoside (nmol)	65.1±6.73	65.3±4.8	30.16±1.92	87.2±2.74
Glucoside carbamate (μmol)	2.48±0.32	1.7±0.1	1.26±0.14	1.51±0.08

Average values and standard deviations. Three week-old plants were treated twice with metolachlor within 48 hr, incubation with BOA was started 24 h after the second metolachlor (N=5) application.

Table 2 UDPG: BOA-6-OH-glucosyltransferase activity

Treatment	Glucosyltransferase Activity (mU/g fresh weight roots)
S_{200} control	17.3±1.07
S_{200} BOA	21.0±0.68
S_{200} control, Metolachlor treated	17.20±0.30
S_{200} BOA, Metolachlor treated	23.25±0.85
S_0 control	18.56±0.48
S_0 BOA	17.85±0.51
S_0 control, Metolachlor treated	15.80±0.34
S_0 BOA, Metolachlor treated	6.97±0.56

Average values and standard deviations. Three week-old plants were treated twice with metolachlor within 48 hr, incubation with BOA was started 24 hr after the second metolachlor application (N=3).

plants, the constitutive glucosyltransferase activity was similar to that determined in roots extracts of S_{200} plants, but BOA incubation induced no increase in activity in sulfur-deficient plants. Application of metolachlor, however, resulted in less enzyme activity in S_0 plants and BOA plus metolachlor cut activity more than 60% (Table 2). *N*-glucosylation was not determined because with protein crude extracts, glucoside carbamate is converted to several other, partly unstable, products that hydrolyze and complicate the determination of enzyme activity.

Analyses of Incubation Media Besides phenoxazinone, AAP and OHPMA were found in most of the incubation media. Furthermore, the incubation media contained MBOA, but none of the known plant detoxification products. Table 3 presents the amounts of AAP, OHPMA, and phenoxazinone accumulation of representative experiments after 24 hr of incubation.

The presence of OHPMA prompted us to elucidate the existence of endophytic fungi in the roots of seedlings and adult plants. As expected, a fungus could be isolated from cut roots. The extent of infection with the fungus varied considerably from root to root. In one sample, *Aspergillus flavus* was found, and in all samples bacteria were found, which were not further determined.

A single fungal strain was identified by microscopic examinations as *F. verticillioides*, formerly known as *F. moniliforme*. Identification on the species level of this *Fusarium* strain was also proved by PCR. Both primer pairs (53-6F/53-6R; FOF1/FOR1) were successfully applied and showed high specificity and selectivity of amplified fungal DNA. PCR with the primer pair 53-6F/53-6R resulted in a specific amplification and generated a

Table 3 Amount of microbial BOA degradation products in the media with accumulation of 2-amino-3H-phenoxazin-3-one

Treatment	2-acetamidophenol (μmol)	*N*-(2-hydroxyphenyl) Malonamic Acid (μmol)	2-amino-3*H*-phenoxazin-3-one (μmol)
S_{200}-plants	0.05±0.02	1.57±0.26	0.08±0.02
Metalochlor treated S_{200} plants	2.1±0.78	3.4±0.98	0.87±0.13
S_0 plants	1.78±0.59	3.05±1.67	0.2±0.14
Metolachlor treated S_0 plants	7.7±4.3	12.77±6.37	0.28±0.08

Time of incubation with BOA (500 μM): 24 h. Average values and standard deviations (N=4).

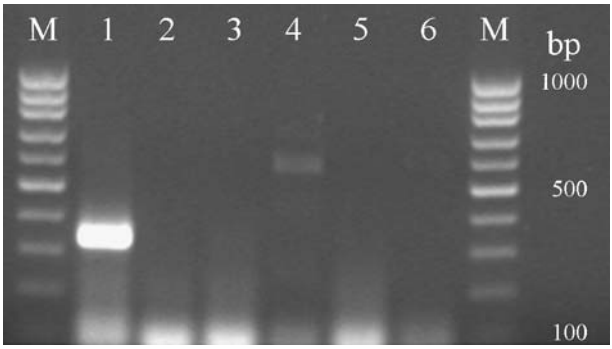


Fig. 2 Polymerase chain reaction amplification products of *Fusarium oxysporum* F-00504 (*lanes 1 and 2*), *Fusarium verticillioides* isolated from maize (*lanes 3 and 4*), and *Fusarium semitectum* (*lanes 5 and 6*) with species-specific primer pairs FOF1/FOR1 (*lanes 1, 3, and 5*) and 53-6F/53-6R (*lanes 2, 4, and 6*)

fragment for the *Fusarium* species originating from maize root tissue determining the strain as *F. verticillioides* (Fig. 2).

As methanolic extracts from the roots neither contained AAP nor OHPMA in detectable amounts, seedlings were incubated with 100 μM solutions of the compounds for 24 hr and for 48 hr to ascertain that absorption of the compounds is negligible. Even after 48 hr, only traces up to 1% of the applied concentration of AAP or OHPMA could be found in the root extracts. Thus, in contrast to BOA, the compounds are absorbed only in traces. Radicle growth tests with the two compounds indicated, however, that traces of OHPMA had a stimulating effect on maize radicle elongation (Fig. 3), whereas AAP had only a significant effect when filter papers were soaked with 250 μM of the compound. Phenoxazinone stimulated maize radicle growth at 250 μM, but had no effect at higher concentrations. The tests were repeated with cress as a representative of dicotyledonous plants. A stimulation of radicle growth was observed with AAP, whereas OHPMA had only a slight effect, and phenoxazinone induced radicle growth at low concentration but was strongly inhibitory at higher concentrations.

Metabolization of AAP, OHPMA, and Phenoxazinone by Fusarium verticillioides To investigate whether the different degradation and detoxification products accumulate over time in the media or whether they are further metabolized by *F. verticillioides*, the fungus was incubated with AAP, OHPMA (both 10 μmol), and 2-amino-3*H*-phenoxazin-3-one (1 μmol). The weight of the mycelia was determined after 2 wk of incubation, and the incubation media was analyzed for the compounds. The fungus was able to use phenoxazinone as a C-source, but growth was reduced (phenoxazinone: 14% in comparison to the control: 100%). Metabolization of phenoxazinone was indicated by loss of the yellowish-orange color of the medium during the incubation time. AAP and OHPMA were both strongly inhibitory to fungal growth (2.3 and 3.4% of the control). AAP, 2.5 μmol, was metabolized, in contrast to OHPMA, where the whole amount applied was regained after 2 wk of incubation.

Experiments with Surface-Sterilized Maize Seedlings

HPLC analysis of the liquid culture medium revealed the presence of plant detoxification products in several samples (Table 4). With *D. stemonitis*, small amounts of gentiobioside carbamate and glucoside carbamate were identified after application of 740 μM BOA. With 100 μM, only glucoside carbamate could be found. Inoculation with *F. verticillioides* led to gentiobioside carbamate and glucoside carbamate accumulation in the medium. When inoculation was performed with both fungi, neither gentiobioside nor glucoside carbamate was

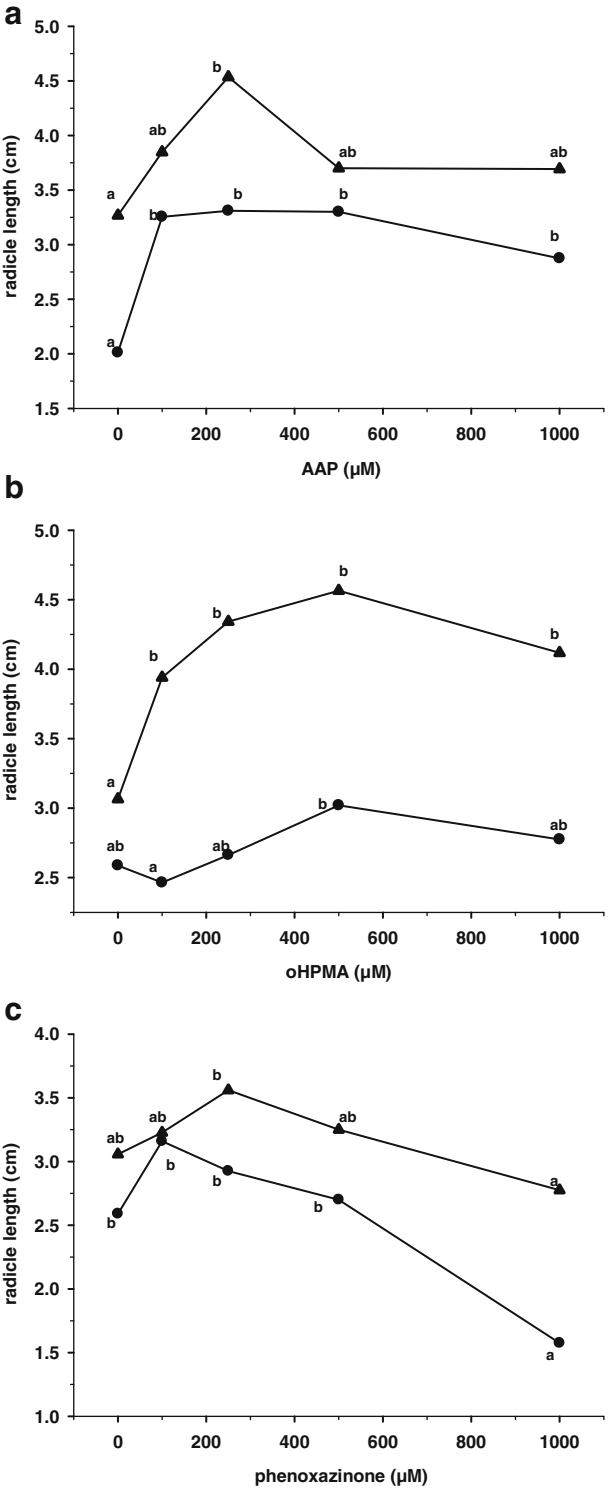


Fig. 3 Radicle growth in presence of AAP (**a**), OHPMA (**b**), and phenoxazinone (**c**). ▲ maize; • cress. Different superscripts for datum points for a particular species denote significant differences ($P<0.05$) (ANOVA SPSS windows version 12.0)

Table 4 Gentiobioside and glucoside carbamate accumulation in the liquid media of sterilized maize plants inoculated with *Doratomyces stemonitis* as nonpathogenic control and *Fusarium verticillioides* after application of 100 or 740 μM BOA

Species μM BOA	Gentiobioside Carbamate (nmol)	Glucoside Carbamate (nmol)
Doratomyces stemonitis		
100 μM BOA	n.d.	181.15±14.15
740 μM BOA	18.3±6.1	69.6±23.2
Fusarium verticillioides		
100 μM BOA	11.45±3.9	90.53±36.8
740 μM BOA	73.9±12.75	478.4±164.8
D. stemonitis/F. verticillioides		
100 μM BOA	n.d.	n.d.
740 μM BOA	n.d.	457.63±64.65

(N=3)

detectable after application of 100 μM BOA. With 740 μM BOA, only glucoside carbamate was found. Surprisingly, none of the samples contained even traces of the microbial degradation products AAP and OHPMA, or phenoxazinone.

Discussion

Although sulfur deficiency results in dramatic growth reductions of the maize plants, BOA-detoxification pathways are not affected, indicating that detoxification of BOA and phytotoxins is important to the plant. The glucosyltransferase involved in BOA-6-OH glucosylation plays an important role in this process. The identification of the enzyme will be published elsewhere.

However, a reduction in BOA detoxification appeared in S_0 plants when metolachlor application was used as an additional stress factor before BOA incubation. In S_{200} plants, metolachlor treatment suppressed an increase of the enzyme activity, but no breakdown appeared. Thus, increasing coincidence of several stress factors results initially in no effect, followed by an increase, and finally, in a reduction of the enzyme activity. The extent and course of time of these reactions should be dependent on plant health, fitness, and genotype.

The analysis of the incubation media demonstrated that a considerable amount of BOA is not absorbed and detoxified by the roots but converted by microorganisms. OHPMA, synthesized via 2-aminophenol, has been described as a characteristic BOA detoxification product of several fungi, e.g., *F. verticillioides* (*F. moliniforme*) *Fusarium subglutinans*, and *Gaeumannomyces graminis* varieties (Friebe et al., 1998; Yue et al., 1998; Glenn et al., 2001, 2003), whereas AAP is supposed to present a branch product of BOA detoxification by *F. verticillioides*. Phenoxazinone, resulting from spontaneous oxidative dimerization of 2-aminophenol, is a known component of BOA degradation initiated by heterocycle cleavage, not only due to fungal but also to bacterial activities. In the incubation studies presented here, *F. verticillioides*, known as an important endophytic fungus of maize (Bacon and Hinton, 1996), is thought to be mainly responsible for OHPMA and at least partly for AAP production, whereas several microorganisms including *F. verticillioides* should be involved in BOA degradation to 2-aminophenol. Thus, endophytic and rhizosphere organisms participate in BOA degradation, which might be regarded as a mechanism that protects the plant, which is important for their own existence. For the plant, energy costs to perform detoxification reactions are lowered, and the time of exposure with harmful effects on biomembranes and cell organelles is shortened.

One of the resulting transformation products, OHPMA was found to be absorbed by maize seedlings only in traces, but the low amounts stimulated maize radicle growth. This compound had only slight stimulatory effects on cress, in contrast to AAP that stimulated cress but maize only at 250 μM. The concentrations used in the bioassays do not present the true concentration of molecules that are in contact with the root. Rather, only molecules within the narrow space of the root absorption tissue are available for uptake. Thus, the true amount of compounds causing stimulation or inhibition of radicle growth should be below the applied concentration. The mechanism of induction is unknown. In this complex interaction, it cannot be excluded that growth stimulation is a secondary effect due to other compounds not related to BOA. Nevertheless, the final effect, a stimulated radicle growth, enhances the chance to escape from BOA-contaminated soil (and to open up new sulfur sources), which is important for further plant growth and associated microorganisms.

Different reactions of cress and maize were also observed with phenoxazinone. This compound was inhibitory for cress only at higher concentrations, while maize radicle growth was not severely affected. Whether endophytic *F. verticillioides* in maize roots may be the cause for the difference is still speculative.

The fungus is a pathogen but can live within the plant without the development of symptoms. As long as the pathogenic character is not manifested, its life as a heterotrophic endophyte is dependent on sustenance by the intact plant. It was unexpected that in the liquid MS medium from sterilized maize seedlings inoculated with *F. verticillioides* and/or *D. stemonitis* that none of the microbial degradation products could be detected. From another study, it is known that the fungi are able to consume exuded plant detoxification products (Voloshchuk et al., unpublished data). In this plant–fungus relationship under sterile conditions, where *F. verticillioides* does not appear as an endophyte, the fungi do not start to degrade BOA not yet absorbed by plants. A possible explanation might be the necessity of bacterial initiation of BOA cleavage. On the other hand, fungi perform the degradation when they are grown in pure culture, a situation in which they are not fed by a plant. Under sterile growth conditions with the plant, a competition with root colonizing bacteria is not occurring. Perhaps the lack of this competition may explain the inactivity of the fungi that require glucosylated plant detoxification products.

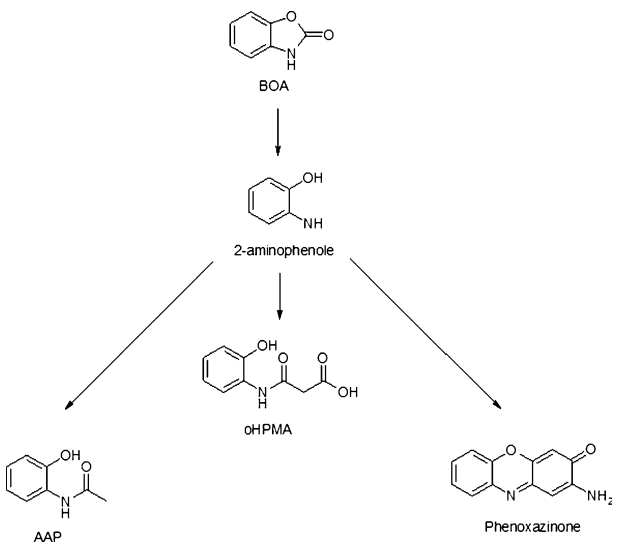


Fig. 4 BOA and its microbial detoxification products

As the media of non-sterile plant incubation with BOA did not contain any plant detoxification products, exuded products also may be consumed by the fungi under non-sterile conditions. In an earlier study with a different experimental arrangement, plant detoxification products (Fig. 1) were exuded after transferring plants to BOA-free medium (Sicker et al., 2001). Thus, the interactions might be more complex when the participation of additional, rhizosphere-colonizing microorganisms is also considered. The complexity of rhizosphere biology has been recently highlighted by Walker et al. (2003).

Our results can be summarized as follows: (1) BOA detoxification is reduced in metolachlor-treated plants suffering from sulfur deficiency. (2) Endophytic *F. verticillioides* and probably other microorganisms participate in BOA detoxification, as indicated by the appearance of the microbial degradation products AAP, OHPMA, and phenoxazinone. *F. verticillioides* seems to be able to degrade phenoxazinone. However, when the medium of sterilized maize plants is inoculated with the fungus, microbial products do not accumulate. (3) AAP and OHPMA are absorbed by maize roots only in traces. OHPMA stimulates maize radicle growth, AAP stimulates cress radicle growth. Phenoxazinone (1 mM) inhibits only cress significantly.

The results imply that allelochemical interactions are affected when maize plants are exposed to combined stress factors. The application of metolachlor likely causes negative effects by weakening detoxification processes in sulfur-deficient plants. Our findings underline the importance of microorganisms involved in the degradation of BOA as partners of a complicated ecological network. The role of endophytic *F. verticillioides* as a supporter in BOA detoxification has to be elucidated by further experiments using plants not infested with the fungus. Plant–fungus interactions seem to be important in plant survival of allelopathic attacks. A disturbance of the sensitive ecological balance by herbicide application can have broad-ranging effects under stress conditions.

Acknowledgements We thank S.C. Pytel from the genetic engineering section of the Institute of Cell Biology and Genetic Engineering NANU, Kiew, Ukraine, for help with the sterile plant cultures and Tom Colby for help with the manuscript. M.S. thanks the BMBF, Internationales Büro for financial support of the cooperation project between the University of Bonn, Germany and the National University of Agriculture, Kiew, Ukraine.

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J Chem Ecol (2007) 33:239–250
DOI 10.1007/s10886-006-9218-5

Role of Catechol Structure in the Adsorption and Transformation Reactions of L-DOPA in Soils

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Received: 6 June 2006 / Accepted: 31 October 2006 /
Published online: 29 December 2006
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Abstract 3-(3′,4′-Dihydroxyphenyl)-L-alanine (L-DOPA), which is synthesized in velvet bean (*Mucuna pruriens*), inhibits plant growth. The concentration of L-DOPA in soil is reduced by adsorption and transformation reactions, which can result in the reduction of its plant-growth-inhibitory activity. To determine which part of the L-DOPA structure is involved in the adsorption and soil transformation reactions, we compared the kinetics of L-DOPA disappearance in a volcanic ash soil with that of L-phenylalanine (3-phenyl-L-alanine) and L-tyrosine (3-(4′-hydroxyphenyl)-L-alanine), compounds that are similar in structure to L-DOPA but do not have a catechol (*o*-dihydroxybenzene) moiety. L-Phenylalanine and L-tyrosine were not adsorbed and transformed in the soil at equilibrium pH values between 4 and 7. These results suggest that the adsorption and transformation reactions of L-DOPA in the soil involve the catechol moiety and not the amino and carboxylic acid groups, which are common to all three compounds. Like L-DOPA, (+)-catechin, another allelochemical that contains a catechol moiety, underwent adsorption and soil transformation reactions. Thus, we concluded that the concentrations of allelochemicals bearing a catechol moiety in soils will decrease rapidly owing to adsorption and transformation reactions, and this decrease will be faster in soils with a high pH value or high adsorption ability. Owing to this decrease in concentration, allelopathic phenomena may not occur.

Keywords Allelopathy · Catechin · *Mucuna* · Velvet bean · Volcanic ash soil

Introduction

Velvet bean (*Mucuna pruriens*) inhibits the growth of neighboring plants by releasing the phytotoxic substance 3-(3′,4′-dihydroxyphenyl)-L-alanine (L-DOPA, Fig. 1) (Fujii et al., 1991, 1992; Fujii, 2000). L-DOPA content in fresh leaves and roots of velvet bean ranges

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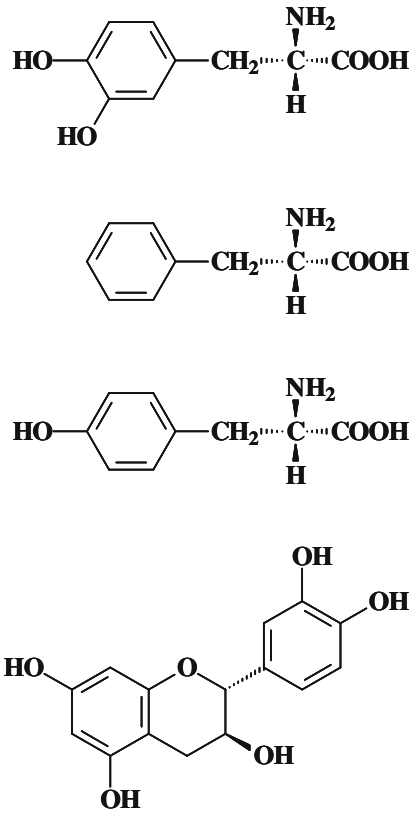


Fig. 1 Chemical structures of 3-(3′,4′-dihydroxyphenyl)-L-alanine (L-DOPA), 3-phenyl-L-alanine (L-phenylalanine), 3-(4′-hydroxyphenyl)-L-alanine (L-tyrosine), and (+)-catechin

from 0.5 to 1.5%, and the yield of fresh leaves and stems ranges from 20 to 30 t ha^{-1} (Fujii et al., 1991). Therefore, velvet bean could produce 100–450 g ha^{-1} (0.05–0.23 mol m^{-2}) of L-DOPA. Because plant-growth-inhibitory activity of L-DOPA in the absence of soil has been observed at concentrations of at least 1×10^{-4} to 1×10^{-5} M (Fujii et al., 1991; Nishihara et al., 2004; Furubayashi et al., 2005; Hiradate et al., 2005), L-DOPA released from velvet beans may inhibit the growth of neighboring plants if L-DOPA persists in soil environments.

Shibuya et al. (1994) observed plant-growth-inhibitory activity of L-DOPA in a granitic sandy soil ("Masa" soil) but not in an Andosol, probably because of the high ability of the Andosol to adsorb L-DOPA. Furubayashi et al. (2005) showed that the concentration of L-DOPA in a soil-water mixture decreases over time, owing to (i) adsorption on the soil, (ii) chemical transformation, and (iii) microbial degradation. Hiradate et al. (2005) reported that the adsorption mechanism involves a ligand exchange reaction in an Andosol, and that the soil components catalytically accelerate the transformation of L-DOPA. Hiradate et al. (2005) also demonstrated that the adsorbed and transformed forms of L-DOPA exhibit no plant-growth-inhibitory activity. Furubayashi et al. (2005) determined the amount of L-DOPA adsorbed and the rate of transformation at equilibrium pH values between 4 and 7 in three soil types (volcanic ash, alluvial, and calcareous soils). The amount of adsorbed L-

DOPA and the rate of transformation at equilibrium pH 6 in the presence of volcanic ash soil were 0.068 mol kg^{-1} soil and 0.057 mol kg^{-1} soil d^{-1}, respectively, and these values indicate that L-DOPA can be expected to disappear rapidly in this type of soil.

L-DOPA contains a catechol moiety, and catechol is easily oxidized in soils, especially under alkaline conditions (Kumada, 1981). Like catechol, L-DOPA may undergo soil oxidation reactions. Many other allelochemicals also contain a catechol moiety. For example, robinetin, myricetin, and quercetin are produced by *Robinia pseudo-acacia* L. (Iqbal et al., 2004); caffeic and chlorogenic acids are present in wild perennial buckwheat (*Fagopyrum cymosum* M.; Tsuzuki and Yamamoto, 1987); digallic and ellagic acids have been detected in soils under the canopy and in the litter of a lowland forest (Lodhi, 1976); (±)-catechins are secreted by spotted knapweed (*Centaurea maculosa*; Bais et al., 2002, 2003); and (+)-catechin has been isolated from buckwheat (Iqbal et al., 2003). All these compounds show plant-growth-inhibitory activity, but their concentrations in soils may be reduced owing to the presence of the catechol moiety in their structures, which we suspect is involved in their adsorption and soil transformation reactions.

In the present study, to elucidate the role of the catechol moiety in L-DOPA adsorption and soil transformation reactions, we investigated the kinetics of the disappearance of L-phenylalanine and L-tyrosine (Fig. 1), which have chemical structures similar to the structure of L-DOPA but lack the catechol moiety, at various equilibrium pH values in a volcanic ash soil. We also investigated whether (+)-catechin underwent adsorption and transformation reactions in a volcanic ash soil and whether the decrease of (+)-catechin concentration in soil solutions corresponded to the reduction of plant-growth-inhibitory activity. We expect this information to be useful in understanding the chemical behaviors of phytotoxic catechol compounds in soils.

Methods and Materials

Soil Samples Samples of a volcanic ash soil (Silandic Andosol, FAO, 1998; Melanudand, Soil Survey Staff, 1999) and an alluvial soil (Anthrosol, FAO, 1998; Aquept, Soil Survey Staff, 1999) were collected from the A_p horizons of an upland agricultural field and a paddy field, respectively, in Tsukuba, Japan (Hiradate and Uchida, 2004). A sample of a calcareous soil (Chromic Luvisol, FAO, 1998; Hapludalf, Soil Survey Staff, 1999) was collected from the A_p horizon of an upland agricultural field in Yomitan, Okinawa, Japan. The soil samples were air-dried and then ground and finely sieved (<0.5 mm).

Analysis of Soil Components Particle size analysis was performed by the pipette method (Day, 1965). Total organic components were determined by a dry combustion method (Sumigraph NC analyzer NC-900, Sumika Chem. Anal. Service, Japan). Determination of soil pH in H_2O was performed with a pH meter with a glass electrode. To determine the pH(H_2O) values, 10.0 g of air-dried soil were mixed with 25 ml of deionized water. The mixture was allowed to stand over 12 hr with occasional shaking, and the pH was measured in the suspension.

Chemicals L-Phenylalanine and L-tyrosine were purchased from Wako Pure Chemical Industries (Osaka, Japan). (+)-Catechin was purchased from Spectrum Chemical (Gardena, CA, USA).

Kinetics of L-Phenylalanine, L-Tyrosine, and (+)-Catechin Disappearance in the Presence of the Volcanic Ash Soil Fifty milligrams of the volcanic ash soil sample (oven-dry basis)

were placed in a 12-ml glass centrifuge tube. Deionized water, 1.0 ml of a 1-M NaCl solution (background electrolyte), 50 μl of chloroform (an antibiotic), and a dilute HCl or NaOH solution (for pH adjustment) were added to the tube to obtain a liquid-phase volume of 3.0 ml and equilibrium pH values between 4 and 7. The tube was capped with a screw-on Teflon-sealed cap and shaken overnight to establish equilibrium. Then, 2 ml of a 5-mM L-phenylalanine or (+)-catechin solution were added to the soil suspension (0.2 mol L-phenylalanine or (+)-catechin kg^{-1} soil). The resulting mixture was shaken at 100 rpm for 1–120 hr at 30°C in the dark. The same procedure was applied to another 50 mg of soil samples, but 0.25 ml of 4 M NaCl and a dilute HCl or NaOH solution were added to the tube to obtain a liquid-phase volume of 1.0 ml and equilibrium pH values between 4 and 7. Then, instead of 2 ml of 5 mM L-phenylalanine or (+)-catechin, 4 ml of 2.5 mM L-tyrosine were added to reach the same final concentration (0.2 mol L-tyrosine kg^{-1} soil).

After filtration of the soil suspension through a cellulose acetate filter membrane (pore size, 0.2 μm; Advantec Toyo Kaisha, Tokyo, Japan), the equilibrium pH was determined with a pH meter with a glass electrode, and the concentration of the test chemical was determined with a high-performance liquid chromatograph (HPLC: pump, L-6200; UV–vis detector, L-4200H; autosampler, AS-2000; column oven, L-5025; chromatogram integrator, D-2500; Hitachi, Tokyo, Japan) equipped with a reversed-phase analytical column (Inertsil ODS-3, 5 μm, 4.6-mm i.d., 250-mm length, GL Sciences, Tokyo, Japan). L-Phenylalanine and L-tyrosine were eluted with a solution of 0.1 M phosphate buffer (pH 2) and methanol (L-phenylalanine, 8:2 v:v; L-tyrosine, 9:1 v:v) at a flow rate of 1 ml min^{-1} at a column temperature of 40°C; the detection wavelength was 200 nm. (+)-Catechin was eluted with a mixed solution of deionized water and methanol (7:3 v:v) at a flow rate of 1 ml min^{-1} at a column temperature of 40°C; the detection wavelength was 200 nm. L-Phenylalanine, L-tyrosine, and (+)-catechin eluted at 6.2, 6.0, and 5.4 min, respectively.

Calculation of L-Phenylalanine, L-Tyrosine, and (+)-Catechin Disappearance at Constant Equilibrium pH Value in the Presence of the Volcanic Ash Soil To adjust accurately the equilibrium pH at the end of the reaction time was difficult because the equilibrium pH of the soil mixture changed gradually over the reaction period, owing to the buffering action of the soil and the reaction of the test chemicals. Therefore, we calculated the amounts of chemicals at equilibrium pH values of 4, 5, 6, and 7 by plotting the relationship between the amounts of the test chemicals and equilibrium pH values of the solutions after filtration through the cellulose acetate membrane. This calculation method is similar to that used for L-DOPA in our previous work (Furubayashi et al., 2005).

Kinetics of L-Phenylalanine and (+)-Catechin Disappearance in the Absence of Soil To clarify the effects of solution pH on the stability of L-phenylalanine and (+)-catechin in the absence of soil, we prepared a 5-ml solution of 2 mM L-phenylalanine or (+)-catechin in a 12-ml glass centrifuge tube in the presence of 0.1 M phosphoric acid–sodium phosphate solution buffered at pH values between 4 and 7. After the tube was shaken at 100 rpm for 1–120 hr at 30°C in the dark, the test solution was passed through a cellulose acetate filter membrane (pore size, 0.2 μm; Advantec Toyo Kaisha). Then, the equilibrium pH and the concentrations of the test chemicals in the filtrate were measured by using a pH meter and an HPLC system, as described above.

Kinetics of L-DOPA Disappearance in the Presence and Absence of Soil We measured the kinetics of the disappearance of L-DOPA in the presence and absence of the volcanic ash

soil in a previous study (Furubayashi et al., 2005) under experimental conditions identical to those used in the present study. The kinetic data from the previous study were used for comparison with the data obtained in this study.

Measurement of the Plant-Growth-Inhibitory Activity of (+)-*Catechin in the Presence of Three Types of Soil* A 500-mg portion (oven-dry basis) of the soil (volcanic ash soil, calcareous soil, or alluvial soil) was placed in each well (15.6-mm i.d., 17-mm high, 1.9-cm^2 growing area) of a 24-well plate (Nunclon, NalgeNunc, Denmark). Then, 1.0 ml of a solution containing 10^{-6}–10^{-2} M (+)-catechin in the presence of 0.75% agar (gelling temperature, 30 to 31°C; Nacalai Tesque, Kyoto, Japan) at 40°C was added to each soil-containing well. After the soil suspension was stirred with a stick and incubated at 30°C for 24 hr in the dark, four lettuce seeds (*Lactuca sativa* cv. Great Lakes 366) were placed on the gelled agar–soil mixture. The lettuce seeds were grown for 72 hr at 20°C in the dark, and the inhibitory activity was determined by measuring root elongation and comparing it with that of the control (without (+)-catechin, but in the presence of the corresponding soil). This experiment was replicated three times.

Results and Discussion

In the presence of volcanic ash soil, L-DOPA disappeared over time at equilibrium pH values between 4 and 7, and in the absence of soil, L-DOPA disappeared only at pH values higher than 7 (Fig. 2). Hiradate et al. (2005) previously reported that L-DOPA disappeared in soils because of adsorption and transformation reactions. In contrast, the amounts of L-phenylalanine and L-tyrosine in the presence and absence of soil remained stable at equilibrium pH values between 4 and 7 during the experimental period (1–120 hr; Fig. 2), which indicates that L-phenylalanine and L-tyrosine were neither absorbed nor transformed by the soil.

In our previous work (Furubayashi et al., 2005), we found that when L-DOPA is incubated with soils, it disappears by means of two chemical processes (Fig. 3). The rapid and sudden reduction of L-DOPA concentration observed during the first 4-hr period was attributed to adsorption. The second process was characterized by a constant rate of disappearance caused by catalytic transformation reactions. The adsorption process ends within 8 hr, and after that time, only the catalytic transformation reactions contribute to the disappearance of L-DOPA (Fig. 3; Furubayashi et al., 2005). The amount of L-DOPA disappearing after 8 hr can be expressed by the following linear equation:

$$Y = aX + b, \tag{1}$$

where Y is the amount of L-DOPA disappeared (mol kg^{-1} soil), a is the rate of L-DOPA disappearance by the transformation reactions (mol kg^{-1} soil hr^{-1}), X is the reaction time (hr), and b is the amount of L-DOPA disappeared by adsorption (mol kg^{-1} soil). The rate of transformation (a) and the amount of L-DOPA adsorbed (b) were calculated by using Eq. 1, and the values obtained were plotted against the equilibrium pH values (Figs. 4 and 5).

The presence of volcanic ash soil increases adsorption of L-DOPA (Fig. 4). The amounts of L-DOPA adsorbed by the soil range from 0.020 to 0.068 mol kg^{-1} soil at equilibrium pH values between 4 and 7, and the amounts increased with increasing equilibrium pH. In

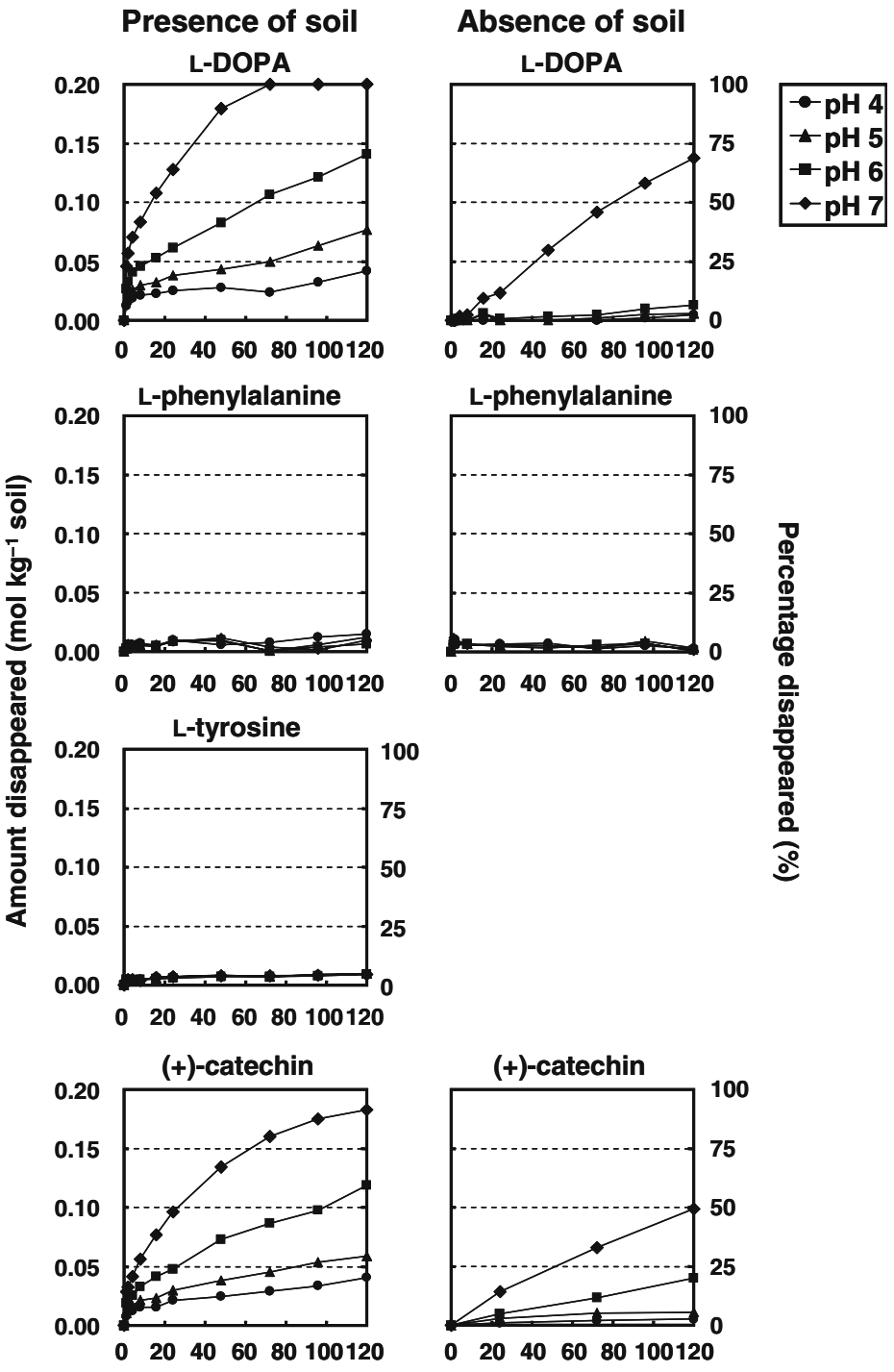


Fig. 2 Kinetics of L-DOPA, L-phenylalanine, L-tyrosine, and (+)-catechin disappearance in the presence (left) and absence (right, excluding L-tyrosine) of a volcanic ash soil at equilibrium pH values of 4, 5, 6, and 7

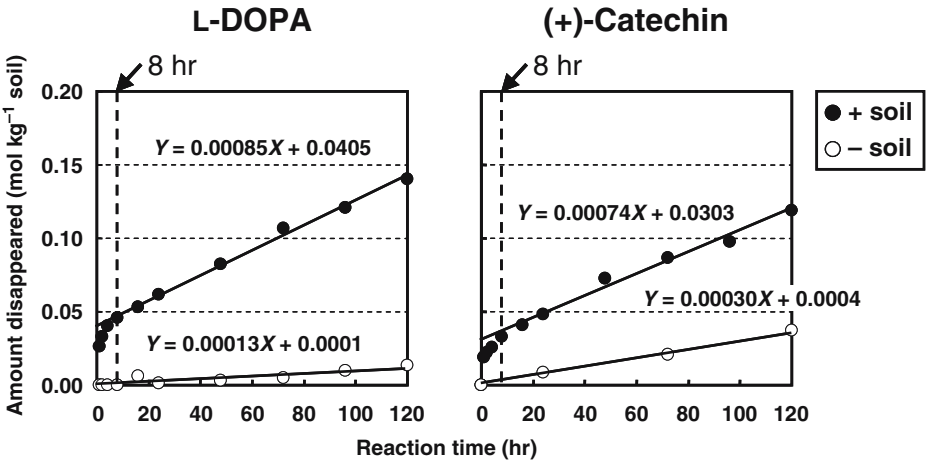


Fig. 3 Determination of the transformation rate and the amount of adsorption of L-DOPA (left) and (+)-catechin (right) using the first-order regression curve $Y=aX+b$, where Y is the amount of L-DOPA or (+)-catechin lost (mol kg^{-1} soil), a is the rate of L-DOPA or (+)-catechin disappearance by transformation (mol kg^{-1} hr^{-1}), X is the reaction time (hr), and b is the amount of L-DOPA or (+)-catechin lost by adsorption (mol kg^{-1}). For the regression analysis, data between 8 and 120 hr of reaction time were used for each series. The reaction experiments were conducted at an equilibrium pH value of 6.0 in the absence (○) and presence (●) of a volcanic ash soil

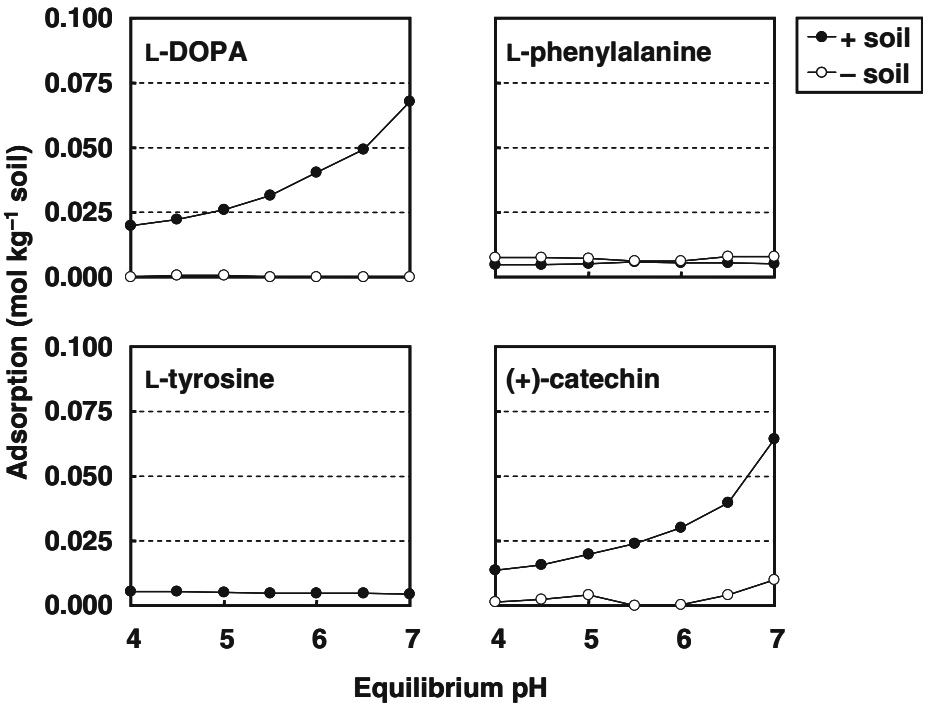


Fig. 4 Adsorption of L-DOPA, L-phenylalanine, L-tyrosine, and (+)-catechin at equilibrium pH values between 4 and 7 in the absence (○) and presence (●) of a volcanic ash soil

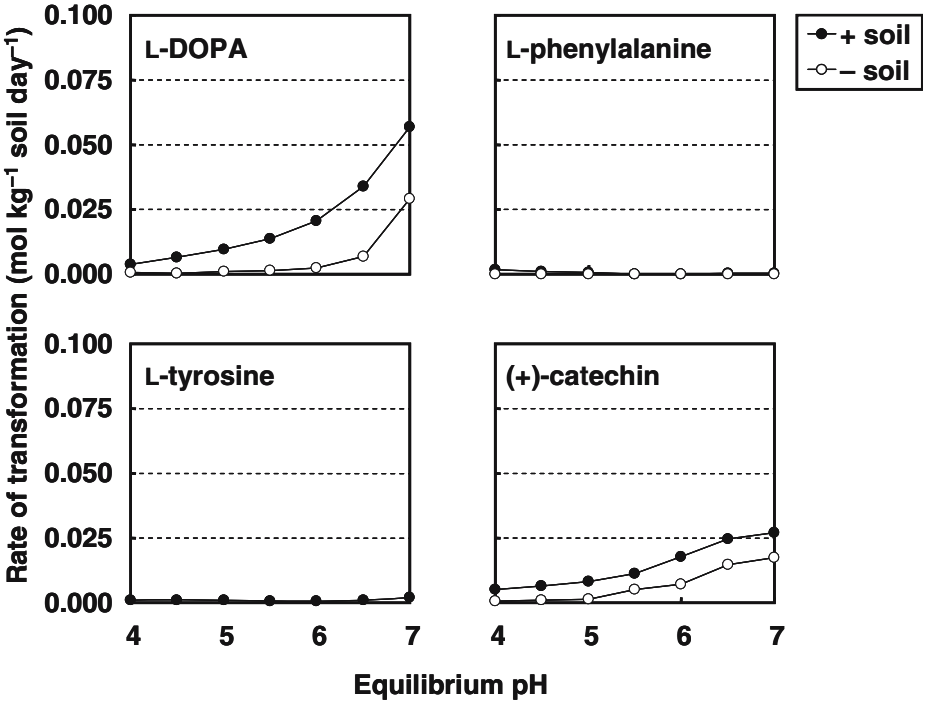


Fig. 5 Transformation rate of L-DOPA, L-phenylalanine, L-tyrosine, and (+)-catechin at equilibrium pH values between 4 and 7 in the absence (○) and presence (●) of a volcanic ash soil

contrast, the amounts of adsorbed L-phenylalanine and L-tyrosine were negligible. The differences in adsorption among the three compounds cannot be attributed to the amino and carboxylic acid groups, which are common to all three structures, and are instead attributable to the number and positions of the hydroxyl group on the benzene rings: L-DOPA has 3′ and 4′ hydroxyl groups, L-tyrosine has a 4′ hydroxyl group, and L-phenylalanine has no hydroxyl groups (Fig. 1). Therefore, we concluded that the 3′,4′-dihydroxyphenyl moiety (that is, the catechol moiety) is responsible for the adsorption.

In both the presence and the absence of soil, the rate of L-DOPA transformation is faster at higher pH values, whereas the transformation rates of L-phenylalanine and L-tyrosine were negligible at equilibrium pH values between 4 and 7 (Fig. 5). In the absence of soil, the rate of L-DOPA transformation is almost zero at equilibrium pH values lower than 6.0, but the rates increase to 0.007 and 0.029 mol kg^{-1} soil d^{-1} at equilibrium pH 6.5, and 7.0, respectively. Hiradate et al. (2005) reported that L-DOPA is automatically transformed into polymerized forms in the absence of soils at equilibrium pH values higher than 6.5 and that this transformation is accompanied by the consumption of OH^-. In the presence of the soil, the L-DOPA transformation rates increase to 0.003 and 0.028 mol kg^{-1} soil d^{-1} at equilibrium pH 4 and 7, respectively. Hiradate et al. (2005) also reported that an Andosol and its components catalytically accelerate the transformation of L-DOPA into heterogeneous components, such as humic substances, as indicated by their spectrum in the proton nuclear magnetic resonance. In contrast, L-phenylalanine and L-tyrosine, which lack the catechol moiety, remained stable both in the absence and in the presence of the

soil, which indicates that the catechol structure, rather than the amino group or the carboxylic acid group, is responsible for L-DOPA adsorption and soil transformation reactions.

L-DOPA content in fresh leaves and roots of velvet bean has been reported by Fujii et al. (1991). If the estimated total production of L-DOPA from velvet bean were added to a soil of 10-cm depth, the maximum amount of L-DOPA in the soil would range from 0.0005 to 0.0023 mol kg^{-1} soil. If a certain amount, close to the above estimated value of 0.0023 mol kg^{-1} soil of L-DOPA, is added to a volcanic ash soil, the concentration of L-DOPA is markedly reduced after 24 hr of reaction time (Fig. 2). Furubayashi et al. (2005) likewise reported that at a concentration of 10^{-3} M (0.002 mol kg^{-1} soil), the plant-growth-inhibitory activity of L-DOPA in the presence of the three soils (volcanic ash, calcareous, and alluvial) is less than 25%. In the presence of these soils, therefore, allelopathic phenomena caused by L-DOPA released from velvet bean may not be observed.

To clarify the influence of the catechol moiety on the adsorption and soil transformation reactions of these compounds, we examined the adsorption and transformation reactions of another catechol-containing allelochemical, (+)-catechin (Fig. 1), both in the presence and in the absence of the volcanic ash soil. When (+)-catechin was incubated with the soil, it disappeared rapidly during the first 4 hr, and then the rate of disappearance slowed and become constant, just as is the case for L-DOPA (Fig. 2). We attributed the disappearance of (+)-catechin in the presence of the soil to adsorption and transformation reactions, as for L-DOPA (Fig. 3). The amount of (+)-catechin adsorbed and the rate of (+)-catechin transformation were calculated by using Eq. 1. (+)-Catechin adsorption on the soil increased with increasing equilibrium pH: 0.013 and 0.054 mol kg^{-1} soil were adsorbed at equilibrium pH 4 and 7, respectively (Fig. 4). The rate of (+)-catechin transformation in the presence of the soil increased with increasing equilibrium pH, to 0.005 and 0.027 mol kg^{-1} soil d^{-1} at equilibrium pH 4 and 7, respectively (Fig. 5). In the absence of soil, the rate of (+)-catechin transformation was negligible at equilibrium pH values lower than 5, but the transformation rate was clearly detected at equilibrium pH values between 5.5 and 7.0 (0.005 and 0.018 mol kg^{-1} soil d^{-1}, respectively). The kinetics of the disappearance of (+)-catechin in the absence and presence of the soil were similar to those of L-DOPA, which indicates again that the presence of the catechol moiety, common to L-DOPA and (+)-catechin, was involved in the adsorption and soil transformation reactions. Oszmianski and Lee (1990) reported that the enzymatic oxidation reaction products of catechin and chlorogenic acid are mainly polymers, and that the production of these is associated with browning. In our study, the gradual change of the (+)-catechin solutions from colorless to yellowish brown likely indicates that (+)-catechin was transformed by oxidative polymerization. Shindo and Huang (1984) found that dihydroxybenzene compounds (hydroquinone, resorcinol, and catechol) are converted into humic acid through semiquinones during the catalytic oxidative reduction of Mn(IV) and Fe(III) oxides. The catechol moieties of L-DOPA and (+)-catechin probably underwent similar reactions in our study.

We investigated the plant-growth-inhibitory activity of (+)-catechin in three soil types (volcanic ash, calcareous, or alluvial). Under natural pH conditions (without pH adjustment, Table 1), (+)-catechin activity became markedly weaker in the presence of soils, whereas in the absence of soils, growth inhibition of at least 50% was observed at initial concentrations higher than 10^{-3} M (Fig. 6). At initial concentrations lower than 3×10^{-3} M (0.006 mol kg^{-1} soil), the inhibitory activity was negligible in the presence of the soils. When 0.006 mol (+)-catechin kg^{-1} soil was incubated with volcanic ash soil at pH 5.8 (no pH adjustment), the (+)-catechin concentration was markedly reduced after 24 hr (Fig. 2). As a result of the

Table 1 Particle size analysis and selected chemical properties of soils used in this study

Soil sample	Particle size analysis			Textural class	Total organic components [a]		pH (H_2O)
	% Sand	% Silt	% Clay		C (g kg^{-1})	N (g kg^{-1})	
Volcanic ash soil	19.7	37.3	43.1	LiC	71.9	4.5	5.8
Calcareous soil	4.2	20.6	75.3	HC	14.9	1.6	7.8
Alluvial soil	41.9	24.4	33.7	LiC	11.0	1.6	5.3

[a] Oven-dry basis

disappearance of (+)-catechin, almost no plant-growth-inhibitory activity was observed. In the environment, allelochemicals are released from living plants at a fairly constant rate during the growth season, and, therefore, the amounts of these chemicals deposited in the soils remain fairly constant. However, adsorption and transformation reactions would prevent the accumulation of the highly toxic allelochemicals L-DOPA and (+)-catechin.

Dalton et al. (1983, 1989) extensively studied the role of clays and organic matter in similar reactions of ferulic acid in soils. These workers found that organic matter was the most active soil component involved in the irreversible retention of ferulic acid in sterile soil. In our study, the clay contents of the soils decreased in the order calcareous soil > volcanic ash soil > alluvial soil, and the organic matter contents decreased in the order volcanic ash soil > calcareous soil > alluvial soil (Table 1). However, we could not confirm the influence of variations in the clay and organic matter contents of the soil on adsorption and transformation reactions of catechin, because the plant-growth-inhibitory activity of (+)-catechin was extremely low in the presence of the soils. In a previous study (Furubayashi

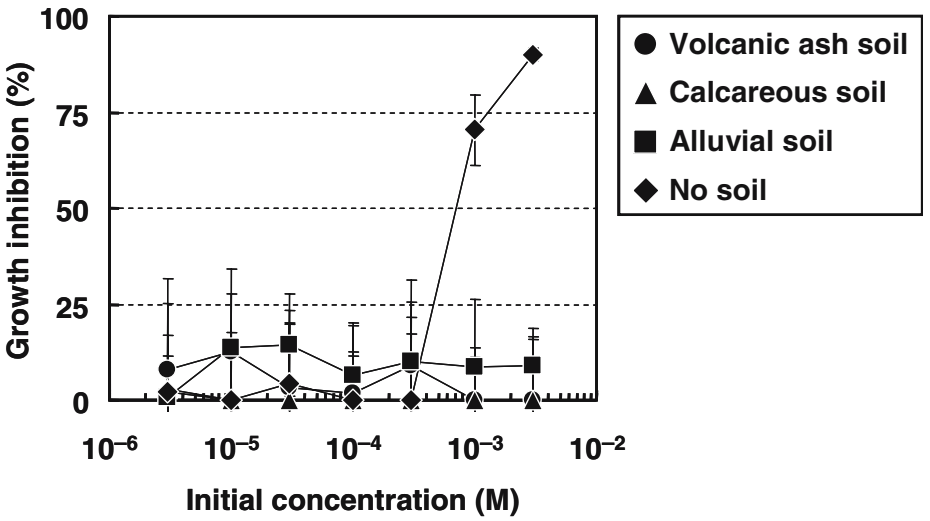


Fig. 6 Inhibitory activity of (+)-catechin, measured in terms of root elongation of lettuce (*Lactuca sativa* cv. Great Lakes 366) grown in agar media in the absence (♦) and presence of soils (●; volcanic ash soil, ▲; calcareous soil, ■; alluvial soil), at various initial concentrations. Inhibition of root growth was detected by measuring root elongation and comparing it with that of the control (with no (+)-catechin, but in the presence of the corresponding soil) after incubation for 72 hr at 20°C in the dark. Bars indicate standard deviation (n=12)

et al., 2005), we observed the largest amount of L-DOPA adsorption in the presence of the volcanic ash soil, which has the highest organic matter content of the three soil types, but the influence of variations in clay content on the adsorption and transformation reactions of L-DOPA was not clear. However, we suspect that the influence may be small because L-DOPA is more polar than ferulic acid and dissolves readily in water. Hiradate et al. (2005) and Furubayashi et al. (2005) have reported that volcanic ash soil, which has high P-retention ability, a high pH (NaF) value, and large amounts of Fe and Al (hydr)oxides, absorbs large amounts of L-DOPA, which suggests that the mechanism of the adsorption of L-DOPA involves a ligand exchange reaction. The mechanisms of the adsorption and transformation reactions of both L-DOPA and (+)-catechin in soils and the effect of variations in clay and organic matter soil contents on their disappearance require further study.

Blum et al. (1987) reported that the retention of ferulic acid (i.e., by adsorption, polymerization, etc.) by clay and organic matter in nonsterile soil is secondary to microbial utilization and uptake by cucumber roots. Likewise, Weidenhamer and Romeo (2004) reported that certain allelochemicals (gallic acid, hydroquinone, arbutin, and benzoquinone) from *Polygonella myriophylla* are degraded rapidly by soil microbes. In our previous study of the behavior of L-DOPA in soil sterilized with chloroform and in nonsterile volcanic ash soil (Furubayashi et al., 2005), we found that L-DOPA disappears after 72 hr, owing to degradation caused by the soil microbes. Therefore, biodegradation by soil microbes, in addition to adsorption and transformation reactions, is a significant factor in the disappearance of L-DOPA and (+)-catechin in soils. In volcanic ash soil environments, the plant-growth-inhibitory activity of L-DOPA and (+)-catechin can be expected to be reduced quickly mainly because of adsorption and transformation reactions that occur rapidly (within 24 hr). The disappearance might be even faster in high-microbial-activity soil than in low-microbial-activity soil because all three processes will occur. The importance of adsorption and transformation reactions relative to that of microbial activity in reducing the concentrations of these compounds in soils also requires further study.

Bais et al. (2003) used methanol to extract natural rhizosphere soil of spotted knapweed in Montana, and estimated that the concentration of (−)-catechin in the soil is about 1.1 mg g^{-1} soil (0.004 mol kg^{-1} soil), and that (−)-catechin inhibits the growth and germination of native species in field soils at natural concentrations. However, they did not report the soil pH, and the extraction method they used was different from our method, making comparison with our results difficult. Under the soil conditions used for the present study, we estimate that the rates of transformation of (+)-catechin were 0.013 and 0.054 mol kg^{-1} soil d^{-1} at equilibrium pH 4 and 7, respectively, and these values indicate that the occurrence of allelopathic phenomena of spotted knapweed in the field would require the release of catechin at the rate of 0.013 mol kg^{-1} soil d^{-1} or more. Blair et al. (2005) demonstrated a rapid decline of catechin concentration within 24 hr after its addition to wet North Carolina soil (Loamy sand/sand, pH 5.2), and those results support ours. We expected, therefore, that the occurrence of allelopathic phenomena caused by catechin and L-DOPA would be low in a soil such as volcanic ash soil, calcareous soil, or alluvial soil at pH values between 4 and 7, because in this range, the rate of transformation of catechin and L-DOPA would be high. Many other phytotoxic allelochemicals contain a catechol moiety (e.g., caffeic acid, digallic acid, ellagic acid, robinetin, myricetin, quercetin, and (−)-catechin). These compounds may disappear rapidly by means of mechanisms similar to those responsible for the disappearance of L-DOPA and (+)-catechin. We conclude that the phytotoxicity of all catechol-bearing compounds released from plants into soils is likely to be substantially reduced by adsorption and transformation reactions, and that the soil concentrations of these

compounds can be expected to be reduced more quickly in soils with high pH values or high adsorption ability. Therefore, allelopathic phenomena may not be observed under such conditions.

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DOI 10.1007/s10886-006-9222-9

Induction of Oxidative Stress by Sunflower Phytotoxins in Germinating Mustard Seeds

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Received: 18 July 2006 / Revised: 15 October 2006 / Accepted: 2 November 2006 /
Published online: 10 January 2007
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Abstract The aim of this study was to investigate the phytotoxic effect of sunflower on physiological and biochemical processes during germination of mustard seeds (*Sinapis alba* L. cv. Nakielska). To exclude the involvement of osmotic stress in seed reaction to phytotoxic compounds, we compared the effect of 10% (w/v) water extract from sunflower (*Helianthus annuus* L. cv. Ogrodowy) leaves and 28.4% (w/v) polyethylene glycol (PEG) 8000 solution characterized by an equal $\Psi = -1$ MPa. We evaluated (1) the amount of hydrogen peroxide (H_2O_2); (2) activities of antioxidant enzymes: superoxide dismutase, catalase, and glutathione reductase; (3) membrane permeability; and (4) level of malondialdehyde (MDA). Both, sunflower compounds and PEG solutions inhibited mustard seed germination, but only phytotoxins caused an increase in the cell membrane permeability, MDA level, H_2O_2 concentration, and alterations in activities of antioxidant enzymes. Our results demonstrate that despite the activation of the antioxidant system by sunflower phytotoxins, reactive oxygen species accumulation caused cellular damage, which resulted in the decrease of germinability and gradual loss of seed vigor. It seems that the negative effect of sunflower on germination of mustard seeds is mostly because of its toxicity and not to its contribution to osmotic potential.

Keywords Antioxidant enzymes · Germination · Mustard · Osmotic stress · Oxidative stress · Phytotoxicity · Sunflower

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Introduction

Allelopathic interactions refer to both natural and agroecosystems. Most effects reported have been identified as being caused by inhibitors of physiological and biochemical processes in plants from chemicals released from living plants into the environment through root exudation, leaching, and volatilization, and passively through decomposition of plant residues. In recent years, a wide selection of plants has been studied for phytotoxic potential. Among them, *Helianthus annuus* L. actively influences the growth of surrounding plants (Irons and Burnside, 1982; Shon and Einhellig, 1982; Leather, 1983; Azania et al., 2003). Phytotoxicity of sunflower residues against *Sorghum vulgare*, *Zea mays*, *Pennisetum americanum*, and *Cyamopsis tetragonoloba* has been reported by Batish et al. (2002). Foliar extracts of sunflower contain chlorogenic and isochlorogenic acids, naphthol derivatives, and scopolin (Wilson and Rice, 1968). In addition, more than other 50 compounds from the active fraction of sunflower extracts have been isolated by Macias et al. (1993, 1996, 1997, 1999, 2000, 2002). Those allelochemicals are mainly terpenoids. Sesquiterpenes are the most abundant, and two new skeletal types, heliannuols and heliespirones, are known.

Like other stress factors, allelochemicals have several molecular targets, and some of their physiological processes or modes of action have been reviewed (Peng et al., 2004; Weir et al., 2004; Gniazdowska and Bogatek, 2005). They include disruption of membrane permeability (Galindo et al., 1999), ion uptake (Lehman and Blum, 1999), inhibition of electron transport in both photosyntetic and the respiratory chain (Peñuelas et al., 1996; Abrahim et al., 2000; Czarnota et al., 2001), alterations of enzyme activities (Cruz-Ortega et al., 1990; Politycka, 1998), and inhibition of cell division (Cruz-Ortega et al., 1988; Anaya and Pelayo-Benavides, 1997). It has also been postulated that allelopathy stress might lead to an imbalance between antioxidant defenses and the amount of reactive oxygen species (ROS), resulting in oxidative stress (Cruz-Ortega et al., 2002; Bais et al., 2003; Romero-Romero et al., 2005).

Although the role of the antioxidant system in plants in response to environmental stresses has received attention (Bowler et al., 1992; Scebba et al., 1998; Yu et al., 2003; Foyer and Noctor, 2005), our understanding about its putative involvement in response to phytotoxic agents is so far not well documented. The scavenging of the superoxide radical $\left(O_2^{\cdot-}\right)$ is achieved through an upstream enzyme, superoxide dismutase (SOD), which catalyses the dismutation of $\left(O_2^{\cdot-}\right)$ to hydrogen peroxide (H_2O_2) and plays a role in protecting cells against the toxic effects of those radicals produced in different cellular compartments (del Rio et al., 2002). Furthermore, the intracellular level of H_2O_2 is regulated by a wide range of enzymes, the most important being catalase (CAT) (Blokhina et al., 2003). Synthesis of SOD and CAT as an adaptive response to oxidative stress has been reviewed (Mittler, 2002). In addition, effective resistance to prolonged stress would necessitate an augmented capacity for ascorbate regeneration, a process at least partly dependent on the tripeptide thiol (glutathione) in higher plants (Foyer et al., 1995). An important role in glutathione oxidation to glutathione disulfate is played by the latter enzyme in the ascorbate–glutathione cycle, glutathione reductase (GR). Transgenic plants, which have threefold higher GR activity than the control, have also increased tolerance to oxidative stress induced by paraquat (Inze and Montagu, 1995).

The phytotoxicity of sunflower extracts makes them good candidates as a source of a natural herbicide against weed species (Azania et al., 2003 and references therein). In this study, we studied the effect of an aqueous extract from sunflower leaves (*H. annuus* L. cv. Ogrodowy) on mustard (*Sinapis alba* L. cv. Nakielska) seed germination, a common dicotyledonous weed species. Extract from sunflower leaves has a low water potential;

therefore, we compared the effect of sunflower water extract and polyethylene glycol (PEG) solution (characterized by an equal $\Psi = -1$ MPa) on mustard seed germination.

In view of the increasing evidence on the role of ROS and the contribution of some antioxidant enzymes (CAT, SOD, and GR) to various physiological processes during stress conditions, it was of a particular interest to investigate whether H_2O_2 and related oxidative damage i.e. lipid peroxidation and membrane injuries, might play a role in seed responses to sunflower phytotoxic compounds.

Methods and Materials

Preparation of Sunflower Extract Leaves from field grown sunflower (*H. annuus* L. cv. Ogrodowy) were harvested at the beginning of the flowering stage. Tissue (leaves) was air-dried, and ground to obtain a fine powder. Leaf powder (10 g dry weight [DW]) was extracted for 24 hr in 100 ml of distilled water at 20°C. The obtained 10% (w/v) extract was filtered through filter paper and used as a source of phytotoxins (Bogatek et al., 2006; Kupidłowska et al., 2006).

Plant Material and Germination Tests Germination assays of mustard seeds (*S. alba* L. cv. Nakielska) were performed by placing seeds (four replicates of 100 seeds each) in 9-cm petri dishes (25 seeds per dish) containing filter paper moistened with distilled water (control), 28.4% (w/v) PEG (−1 MPa) solution or 10% (w/v) sunflower extract (−1 MPa) at 20°C in darkness. Seeds were considered to have germinated when the radicle had pierced the envelope. Dry matter of mustard seeds was determined by oven drying at 105°C for 48 hr.

Viability Seed viability was estimated with a tetrazolium test. Seeds were cut longitudinally and incubated in 1% solution of 2,3,5-triphenyltetrazolium chloride for 2 hr at 20°C in darkness (Moore, 1973). Seeds in which the embryo exhibited no carmine staining were scored as nonviable.

Biochemical measurements were carried out on mustard seeds after 18 hr and 2, 4, 6, and 8 d of treatment with sunflower extract. For the control and seeds germinated in the presence of 28.4% (w/v) PEG (−1 MPa), measurements were carried out only after 18 hr of germination because longer durations of imbibition allowed radicle protrusion (seeds in all combinations were at the same physiological stage).

H_2O_2 Concentration Hydrogen peroxide contents of seeds were determined according to the method described by O'Kane et al. (1996). Mustard seeds (0.5 g, fresh weight [FW]) were ground with a mortar and pestle in liquid nitrogen. Samples were homogenized with 10 ml of perchloric acid (0.2 M). After 15 min of centrifugation at 11,000×*g* at 4°C, the resulting supernatant was neutralized to pH 7.5 with KOH (4 M), and then centrifuged at 5,000×*g* for 3 min at the same temperature to remove insoluble potassium perchlorate. The obtained supernatant was used for spectrophotometric determination of H_2O_2. The reaction mixture contained 50 µl of the collected supernatant, 12.5 mM of dimethylaminobenzoic acid, 1.25 mM of 3-methyl-2-benzothiazolidone hydrazone, and 20 µl (0.25 U) of horseradish peroxidase for a total volume of 1.5 ml. The reaction started with the addition of peroxidase. Increase in absorbance at 590 nm was monitored after 5 min at 25°C and compared with the absorbance obtained with known amounts of H_2O_2, detected for 5 min at 590 nm. Results are expressed as micromoles per gram of DW and correspond to the means of the values ± SD obtained with three replicates from three independent experiments.

Electrolyte Leakage Germinated seeds (on water, PEG solution, or extract from sunflower leaves) were rinsed with distilled water, and solute leakage was determined. Twenty seeds were placed in 15 ml of distilled water at 20°C in darkness. Conductivity in the medium was measured with a conductivity meter after 2 hr of incubation period. Results are expressed as percent of total leakage from seeds boiled for 20 min in water and represent the means ± SD of the values obtained with three measurements carried out on five replicates.

MDA Content Lipid peroxidation was estimated by measuring spectrophotometrically malondialdehyde (MDA) contents of seeds. The tissue (five seeds) was ground in 5 ml distilled water and homogenized with an equal volume of 0.5% (w/v) 2-thiobarbituric acid in 20% (w/v) trichloroacetic acid. The homogenate was incubated for 30 min at 95°C in a water bath and then cooled quickly on ice and centrifuged at 16,000×*g* for 30 min. The supernatant was used for MDA determination according to Heath and Parker (1968) as described by Bailly et al. (1996). MDA equivalent was calculated from the difference in absorbance at 532 and 600 nm using an extinction coefficient of 155 mM^{-1} cm^{-1}. Results are expressed as nanomoles per gram of DW and represent the mean of 3 replicates ± SD.

SOD, CAT, and GR Extraction Enzyme activities were determined from the extract prepared according to the method of Bailly et al. (1996). All steps of the extraction were carried out at 4°C. Seeds (1 g FW) were homogenized in a cold mortar in 10 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing 2 mM α-dithiothreitol, 0.1 mM EDTA, 1.25 mM PEG 4000, and 0.2 g polyvinylpyrrolidone and mixed for 15 min. The homogenate was centrifuged at 11,000×*g* for 15 min, and the supernatant was filtered through Miracloth, desalted on a PD 10 column (Amersham Biosciences). The obtained extract was immediately used for determination of enzyme activities.

Total SOD (EC 1.15.1.1) Activity of SOD was determined by inhibition of *p*-nitroblue tetrazolium chloride (NBT) photoreduction (Giannopolitis and Ries, 1977). The assay was conducted at 25°C in a total volume of 3 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing 1.3 μM riboflavine, 13 mM methionine, 63 μM NBT, and 50 μl of enzymatic extract, as described by Bailly et al. (1996). Absorbance was monitored at 560 nm after 15 min of illumination, using a spectrophotometer Pharma Spec UV-1700 (Shimadzu, Japan). One unit of SOD is defined as the amount of enzyme that inhibits NBT photoreduction to blue formazan by 50%, monitored at 560 nm. Superoxide dismutase activity of the extracts was expressed as units of SOD per milligram of protein.

CAT (EC 1.11.1.6) Catalase activity was determined according to Clairbone (1985) with some modifications (Bailly et al., 1996). The assay was carried out in a total volume of 3 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 37.5 mM of H_2O_2 and 200 μl of enzyme extract. The decrease in absorbance at 240 nm because of degradation of H_2O_2 ($\varepsilon = 39.4\ mmol^{-1}\ cm^{-1}$) *was monitored every 3 sec for 2 min, using a spectrophotometer, Pharma Spec UV-1700 (Shimadzu). Catalase activity was expressed as nanomoles of* H_2O_2 *decomposed per milligram of protein per minute.*

GR (EC 1.6.4.2) Glutathione reductase activity was determined in 800 μl of 0.1 M potassium phosphate buffer (pH 7.8) containing 0.5 mM β-nicotinamide adenine dinucleotide 2′-phosphate, reduced form (2′-NADPH); 10 mM glutathione, oxidized form; 3 mM $MgCl_2$; and 50 μl enzymatic extract (Bailly et al., 1996). The decline in absorbance

because of NADPH oxidation was recorded at 340 nm every 30 sec for 6–8 min using a spectrophotometer, Pharma Spec UV-1700 (Shimadzu). Glutathione reductase activity was expressed as nanomoles of NADPH oxidized per milligram of protein per minute. All results correspond to the means ± SD of the values obtained with three measurements carried out in three independent experiments.

Protein Determination Protein content in the enzymatic extracts was determined by using a Bradford reagent (Bradford 1976). Bovine serum albumin (Sigma) was used as a calibration standard.

Measurement of Water Potential WP4 Dewpoint Potential Meter (Decagon Devices, Inc., Pullman, WA, USA) was used to measure water potential (Ψ) of 10% (w/v) sunflower extract and 28.4% (w/v) PEG 8000 solution. Samples of both solutions were monitored at 20°C. Results correspond to the means ± SD of the values obtained with three measurements carried out for three of each independent prepared solutions.

Statistical Analysis The values of germinability and viability were represented as means ± SE from four independent experiments, whereas the remaining data were means ± SE from three replicates from three independent experiments. Significant differences between values of H_2O_2 concentration, membrane injury, MDA concentration, and enzyme activities, which were measured in dry mustard seeds and germinating mustard seeds in the presence of water, PEG solution, and sunflower extract, were determined at $P \leq 0.05$ according to Duncan's multiple range test.

Results

Germination and Viability Almost all (95%) mustard seeds germinated on water (control) within 3 d at 20°C and produced typical etiolated seedlings 7–8 cm long after 8 d (Fig. 1).

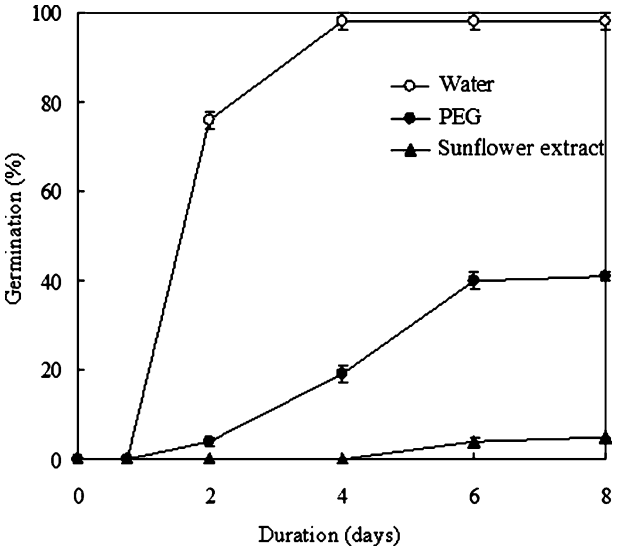


Fig. 1 Mustard seed germination in the presence of water, PEG, and sunflower extract. Data are means ± SD from four independent experiments

The water potential of 28.4% (w/v) PEG equaled that of the 10% (w/v) sunflower extract and reached −1 MPa. Germination on 28.4% PEG solution (Ψ=−1 MPa) was inhibited by almost 50%; after 8 d of incubation on PEG solution, only 48% of seeds germinated and produced etiolated seedlings 5–6 cm long (Fig. 1). The water extract from sunflower leaves (10% w/v, −1 MPa) strongly affected germination of mustard seeds. After 8 d of exposure to phytotoxic compounds, only 5% of seeds germinated (Fig. 1) and failed to develop into normal seedlings (data not shown). For the duration of the test, in the presence of sunflower toxins, no radicles pierced the seed envelope, nor was seedling growth noticed. Those seeds, throughout the course of the experiment, remained at the stage comparable to seeds immersed in water after 18 hr. Therefore, seeds after 18 hr (0.75 d) of imbibition in water were regarded as a potential control for comparative purposes, owing to their similar morphological and physiological growth stage to treated seeds.

Results of the tetrazolium test (Fig. 2) showed that almost all seeds incubated on water were viable. During the time of germination, control seed viability was not changed and we obtained 95% viable seeds (Fig. 2). Similarly, the viability of seeds treated by PEG reached 92% and was constant till the end of the experiment (Fig. 2). During the first 4 d of germination in the presence of sunflower extract, 84% of the seeds remained viable (Fig. 2), although they did not germinate (Fig. 1). A longer treatment with sunflower extract was lethal, and the tetrazolium test showed that seed viability decreased progressively to 65% at the eight day and to 2% of viable seeds after 14 d (Fig. 2).

H_2O_2 Concentration Hydrogen peroxide concentration (Fig. 3) in dry seeds and seeds imbibed with water was 0.11 and 1.33 μmol g^{-1} DW, respectively. Similar values of H_2O_2 level (1.05 μmol g^{-1} DW) were observed in seeds germinated on PEG solution (Fig. 3). Sunflower phytotoxic compounds increased H_2O_2 concentration in mustard seeds. After 18 hr, it was more than three times as high (3.9 μmol g^{-1} DW) as in the control and seeds imbibed on PEG, and increased progressively, reaching 5.78 μmol g^{-1} DW at the fourth day and 7.12 μmol g^{-1} DW at the eight day (Fig. 3).

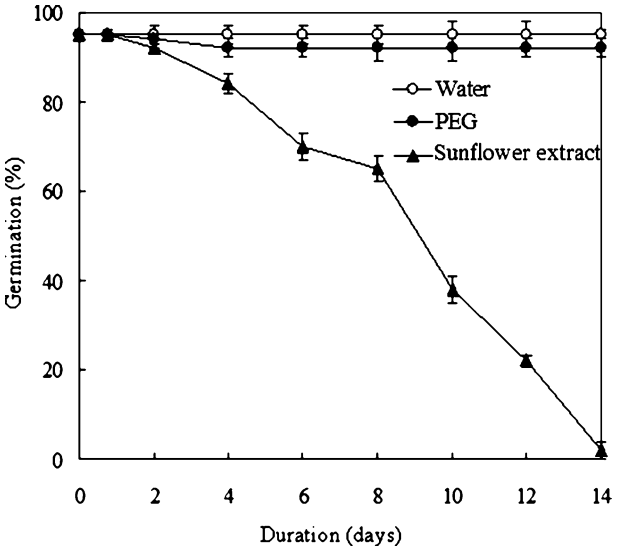


Fig. 2 Viability of mustard seeds in the presence of water, PEG, and sunflower extract. Data are means ± SD from four independent experiments

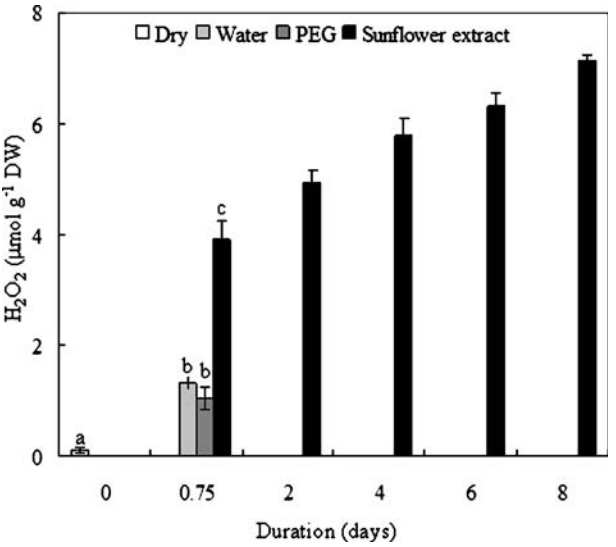


Fig. 3 H_2O_2 concentration in dry mustard seeds and germinating mustard seeds in the presence of water, PEG, and sunflower extract. Data are means ± SD from three replicates from three independent experiments. Significant differences *a*, *b*, and *c* at the 5% level between mustard seeds germinated in the presence of water, PEG solution, and sunflower extract ($P \leq 0.05$, according to Duncan's multiple range test)

Electrolyte Leakage Ion leakage (Fig. 4) from dry mustard seeds or after 18 hr of imbibition on water and PEG solution was low (0.5, 4, and 5% of total leakage, respectively). Treatment of seeds by sunflower extract led to a rapid and marked increase in electrolyte leakage. It represented 19.5% of total leachable electrolytes after 18 hr and increased continuously during the experiment. After 8 d of incubation of mustard seed in the presence of sunflower compounds, electrolyte leakage reached 33% of total electrolyte leakage (Fig. 4).

MDA Content The level of MDA (Fig. 5) in dry seeds was 1.3 nmol g^{-1} DW. After 18 hr of imbibition on water, PEG solution, or sunflower compounds, the MDA content of mustard

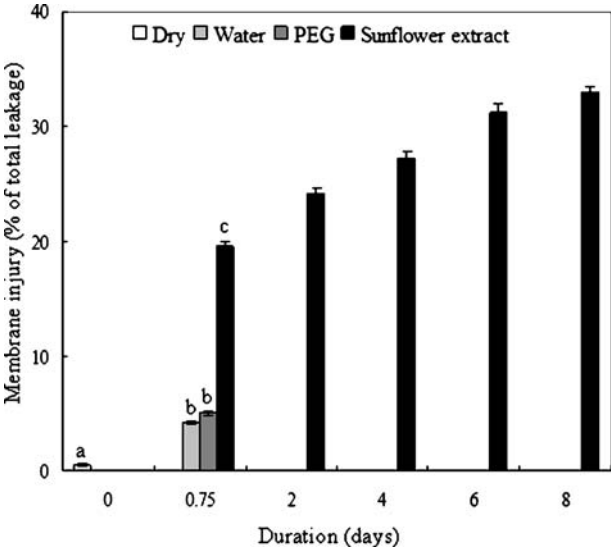


Fig. 4 Membrane injury in dry mustard seeds and germinating mustard seeds in the presence of water, PEG, and sunflower extract. Data are means ± SD from three replicates from three independent experiments. Significant differences *a*, *b*, and *c* at the 5% level between mustard seeds germinated in the presence of water, PEG solution, and sunflower extract ($P \leq 0.05$, according to Duncan's multiple range test)

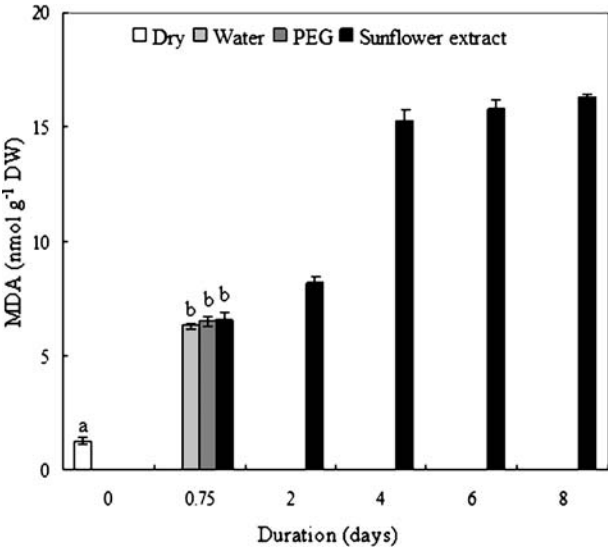


Fig. 5 Malondialdehyde (MDA) concentration in dry mustard seeds and germinating mustard seeds in the presence of water, PEG, and sunflower extract. Data are means ± SD from three replicates from three independent experiments. Significant differences *a* and *b* at the 5% level between mustard seeds germinated in the presence of water, PEG solution, and sunflower extract ($P \leq 0.05$, according to Duncan's multiple range test)

seeds increased to ca. 6.3, 6.5, and 6.6 nmol g^{-1} DW, respectively. Prolonged phytotoxic stress was associated with an increase of MDA concentration until the fourth day, and it remained at a constant value, close to 15.3 nmol g^{-1} DW till the end of the experiment.

Activities of Antioxidant Enzymes Superoxide dismutase activity (Table 1) in dry mustard seeds was 4.61 U mg^{-1} protein. Eighteen hours of imbibition on water, PEG solution, or sunflower extract resulted in an increase of SOD activity to values 7.28, 6.60, and 7.32 U mg^{-1} protein, respectively. The prolonged treatment by sunflower extract increased SOD activity, and after 8 d of germination it was almost doubled, compared to seeds in the imbibed in water, and reached a value 12.11 U mg^{-1} protein.

Table 1 Antioxidant enzyme activities in dry mustard seeds and germinating mustard seeds in the presence of water, PEG solution, and sunflower extract

Combination	Enzyme Activity			
	Days	SOD (units mg^{-1} protein)	CAT (nmol H_2O_2 mg^{-1} protein min^{-1})	GR (nmol NAPDH mg^{-1} protein min^{-1})
Dry	0	4.61±0.14[a]	0.01±0.005	ND
Water	0.75	7.28±0.22[a]	0.95±0.03	2.11±0.06[a]
PEG	0.75	6.60±0.20[a]	1.34±0.04	1.84±0.05[a]
Sunflower extract	0.75	7.32±0.22[a]	1.17±0.03	0.31±0.01[a]
	2	9.33±0.28	1.84±0.05	1.37±0.04
	4	11.03±0.33	4.63±0.09	1.52±0.05
	6	11.33±0.34	6.03±0.12	1.64±0.05
	8	12.11±0.36	9.17±0.27	2.06±0.06

Data are means ± SD from three replicates from three independent experiments.

[a] Significant differences at the 5% level between mustard seeds germinated in the presence of water, PEG solution, and sunflower extract ($P \leq 0.05$, according to Duncan's multiple range test).

ND = not detected

Catalase activity (Table 1) in dry seeds was 0.01 nmol H_2O_2 mg^{-1} protein min^{-1}. After 18 hr of imbibition, it increased similarly in each three combinations (control, PEG solution, and sunflower extract), reaching values of 0.95, 1.34, and 1.17 nmol H_2O_2 mg^{-1} protein min^{-1}, respectively. The prolonged seed incubation on sunflower extract resulted in a further increase of CAT activity. After 8 d of exposure to phytotoxins, it achieved 9.17 nmol H_2O_2 mg^{-1} protein min^{-1}.

Glutathione reductase activity (Table 1) in dry mustard seeds was not detected but during 18 hr of imbibition on water and PEG solution it increased significantly (2.11 and 1.84 nmol NADPH mg^{-1} protein min^{-1}, respectively). However, GR activity in seeds imbibed 18 hr on sunflower extract was eight times lower than that measured in control or seeds treated by PEG solution. Subsequent imbibition of seeds imbibed in the present of sunflower toxins resulted in increase of GR activity. After 8 d of phytotoxin treatment, it increased to a value similar to that observed in the control and osmotic stressed seeds after 18 hr of incubation, and reached 2.06 nmol NADPH mg^{-1} protein min^{-1}.

Discussion

The most widely used biological assays for the action of phytotoxins are seed germination and seedling growth studies. The delay and reduction of seed germination and/or inhibition of root and shoot growth are usually the first visible symptoms of phytotoxic stress (Dayan et al., 2000). It was previously demonstrated that sunflower produces different allelochemicals that exhibit phytotoxicity against many plant species (Macias et al., 1999, 2002) and, therefore, might be used as an alternative strategy for weed control (Leather, 1983, Azania et al., 2003). Our previous study confirmed that the effect of aqueous sunflower foliar extracts on mustard seed germination is dose-dependent (Bogatek et al., 2006). Germination of mustard seeds is almost fully inhibited by 10% (w/v) water extract of sunflower leaves (Fig. 1). The osmotic potential of sunflower extract used in our experiment was determined to be close to −1 MPa, and for that reason PEG solution characterized by the same osmotic potential was used to determine whether the inhibition of germination resulted from limited water availability. Imbibition of seeds on PEG solution did not fully inhibit their germination but delayed radicle protrusion and finally allowed 48% of seeds to germinate (Fig. 1). Thus, the inhibitory effect of sunflower foliar extracts on germination cannot be explained by a limitation in water availability, but rather by a toxic effect of the extract, as shown by the viability test (Fig. 2).

Many identified allelochemicals inhibit or delay seed germination. (−)-Catechin, the allelochemical from root exudates of *Centaurea maculosa* inhibited germination of *Festuca idahoensis* and *Koelaria micrantha* (Bais et al., 2003). Different phenolic compounds (vanillic, *p*-coumaric, and protocatechuic acids) used at concentrations from 10^{-5} to 10^{-6} M reduced germination of *Pinus laricio* seeds (Musculo et al., 2001). Capsaicin isolated from *Capsicum* spp. at the concentration 10–90 mM suppressed germination of lettuce (*Lactica sativa*), crabgrass (*Digitaria sanguinalis*), timothy (*Phleum pretense*), and ryegrass (*Lolium multiflorum*) (Kato-Noguchi and Tanaka, 2003–2004). Although allelochemicals act as inhibitors of seed germination, their mode of action often remains unclear. Recently, it was proposed that oxidative stress could play a role in phytotoxic phenomenon (Weir et al., 2004). The exposure of cucumber roots to ferulic and *p*-coumaric acids resulted in increased H_2O_2 concentration in the tissue (Politycka, 1996). Bais et al. (2003) showed that

(−)-catechin elicits a burst of ROS in susceptible plant roots, and the kinetics of ROS induction was similar to the patterns of cell death induced by (−)-catechin. The toxicity of many quinones and phenols can largely be attributed to the formation of semiquinone radicals that donate electrons to molecular oxygen, forming superoxide anions $\left(O_2^{\cdot-}\right)$ (Weir et al., 2004). Our results show that an increase in H_2O_2 content occurs early during mustard seed imbibition in sunflower extract (Fig. 3). We demonstrate that the detrimental effect of sunflower on germination of mustard seeds is not strictly associated with water stress (comparison of PEG and phytotoxins treatment) but rather with the imposition of a precocious oxidative stress.

One of the best known cellular targets of oxidative stress is lipid peroxidation, which is a free-radical chain process leading to the deterioration of polyunsaturated fatty acids (PUFAs). Lipid peroxidation is likely to degrade PUFAs present in membranes, leading to severe damage to membranes or to reserve lipids of oily seeds (Scandalios, 1993; Foyer et al., 1994). One of the indicators of plasma membrane damage is electrolyte leakage measurement. After 8 d of incubation of mustard seed on sunflower compounds, ion leakage reached 35% of total leachable electrolytes, thus suggesting damage to plasma membranes (Fig. 4). This observation is in agreement with that obtained with cucumber cotyledons treated with natural sesquiterpenolide, dehydrozaluzanin C, causing a dramatic and rapid plasma membrane leakage (Galindo et al., 1999). In the present paper, symptoms of membrane injuries occurred concomitantly to H_2O_2 accumulation (Fig. 3) and preceded an increase in the content of MDA, a compound that reveals the occurrence of measurable processes of lipid peroxidation (Fig. 5). Lipid peroxidation, therefore, seemed to occur when seeds started to die, i.e., after 4 d of treatment by phytotoxins (Fig. 2), and thus refers to deep damage of the seed lipid component that may concern either membrane or reserve lipids. Storage materials in mustard seeds are mainly proteins (ca. 60%) and lipids (ca. 40%), depending on the variety and environmental conditions. Increased lipid peroxidation in the presence of phytotoxins has also been shown in tomato roots (Cruz-Ortega et al., 2002) and in cucumber roots (Politycka, 1996). Damage of the whole membrane system of different plants (rape, cucumber, corn, and sorghum) analyzed at the biochemical and ultrastructural level has been detected in the presence of secalonic acid F, produced by *Aspergillus japonicus* (Zeng et al., 2001). Allelochemicals can damage cell membranes through direct interaction with a constituent of the membrane, or can result in an impairment of some metabolic processes necessary for the maintenance of membrane function.

With regard to the possible roles of oxidative stress in phytotoxicity, the changes in the main antioxidant enzymes have been investigated during seed imbibition on sunflower leaf extract. During the first 2 d of sunflower treatment, SOD and CAT activities did not evolve significantly and were similar to those measured in seeds imbibed in water (Table 1). However, GR activity was transiently and dramatically reduced by sunflower foliar extract (Table 1), which may account, at least in part, for the increase of H_2O_2 in the treated seeds (Fig. 3). Glutathione reductase is known to play a role in the control of endogenous H_2O_2 through its participation in the ascorbate–glutathione cycle and through the formation of reduced glutathione, a compound able to scavenge ROS (Goel et al., 2003). Its impairment would alter the scavenging ability of the cellular ROS and lead to an unbalanced accumulation of H_2O_2. Longer treatment of seeds by allelochemicals, i.e., from the fourth to eight day, was associated with a stimulation of CAT and SOD activities and with the recovery of GR activity (Table 1). Indeed, ROS are known to regulate the expression of many genes. One commonly cited possibility concerns the activation of transcription factors by redox status changes (Foyer and Noctor, 2003). Alternatively, gene

promoter regions may possess antioxidant response elements suspected to play a role in either H_2O_2 or antioxidant sensing, as in the case of the maize CAT *Cat1* gene (Scandalios et al., 1997). We cannot exclude the possibility that sunflower phytotoxins may influence synthesis of CAT isoforms. Western blot analysis showed that enhancement of CAT activity is associated with synthesis of CAT 5 and CAT 6 isoforms in seeds treated by sunflower extract (Oracz, personal communication). The results provide evidence that the effect of phytotoxins is not direct but occurs through regulation of ROS level in cells of mustard seeds. However, this stimulation of the cell antioxidant system was insufficient to avoid H_2O_2 accumulation (Fig. 3) or to prevent lipid peroxidation (Fig. 5) and did not prevent cell death (Fig. 2). Our data are consistent with those of Yu et al. (2003), which showed that exposure of cucumber roots to phytotoxic compounds significantly increased SOD activity. An increased CAT activity in response to allelochemicals has also been demonstrated in germinating cucumber seeds treated by different benzoic acids (Maffei et al., 1999). Romero-Romero et al. (2005) observed increased CAT activity in tomato affected by phytotoxins from *Sicyos deppei*. However, regarding the complex composition of sunflower leaves extract (Macias et al., 2002) it is not possible to conclude that there is a specific effect of phytotoxic compounds on antioxidant enzymes. Furthermore, it is known that activity and expression of most antioxidant enzymes is stimulated by ROS accumulation (Vranova et al., 2002; de Gara et al., 2003; Apel and Hirt, 2004). The first effect of sunflower phytotoxicity is a dramatic increase in H_2O_2 concentration and an inhibition of GR activity. It is thus highly probable that the observed stimulation of antioxidant enzymes does not result from a direct effect of sunflower compounds but is rather a consequence of the oxidative burst that occurs during the first hours of seed imbibition.

The presented data show that although some disturbances in seed metabolism during the germination process are common in both phytotoxic and osmotic stress, the markedly different response is a dramatic increase in the concentration of H_2O_2 and changes in GR activity. Therefore, we conclude that despite the similar effect caused by both stresses (inhibition/delay of germination), the mode of their action is different at the biochemical level, as was previously suggested by Romero-Romero et al. (2005).

Our results bring together several lines of evidence, indicating that the deleterious effect of phytotoxins on germination may occur through the imposition of an oxidative stress. The specific effect of sunflower compounds on GR activity explains, in part, the accumulation of H_2O_2, but future studies will have to deal with the precise mechanisms of ROS production during this treatment.

Acknowledgements We thank Stewart A. Brown, Professor Emeritus of Trent University, Peterborough, Ontario, Canada for assistance in preparing the English version of the manuscript.

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J Chem Ecol (2007) 33:265–275
DOI 10.1007/s10886-006-9227-4

L-DOPA Increases Lignification Associated with *Glycine max* Root Growth-Inhibition

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Received: 10 April 2006 / Revised: 25 October 2006 / Accepted: 6 November 2006 /
Published online: 29 December 2006
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Abstract L-3,4-dihydroxyphenylalanine (L-DOPA), an allelochemical exuded from the roots of velvet bean [*Mucuna pruriens* (L.) DC. var. *utilis*], presents a highly inhibitory action to plant growth. The effects of L-DOPA on phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and peroxidase (POD, EC 1.11.1.7) activities, and phenolic compound and lignin content in soybean [*Glycine max* (L.) Merr.] roots were investigated to determine the possible phytotoxic mechanism. Three-day-old seedlings were cultivated in half-strength Hoagland nutrient solution (pH 6.0), without or with 0.1 to 1.0 mM L-DOPA in a growth chamber (25°C, 12-hr light to 12-hr darkness photoperiod, irradiance of 280 μmol m^{-2} s^{-1}) for 24 hr. In general, the length, fresh weight, and dry weight of the roots decreased, whereas PAL and POD activities and phenolic compound and lignin content increased after L-DOPA treatments. Results showed the susceptibility of soybean to L-DOPA and reinforce the role of this nonprotein amino acid as a strong allelochemical. The present findings also suggest that L-DOPA-induced inhibition in soybean roots may be because of a cell wall stiffening process related to the formation of cross-linking between cell wall polymers linked to lignin production.

Keywords Allelopathy · L-DOPA · Lignin · Peroxidase · Phenolic compounds · Phenylalanine ammonia-lyase · Root growth · Soybean · Nonprotein amino acid

Introduction

For years, it was known that plants release organic compounds into the environment from their aerial or subaerial parts as exudates, volatiles, and/or decomposition residues. These compounds may accumulate in the soil environment and affect the growth and development

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of neighboring plants, an interaction called allelopathy (Einhellig, 1995). Velvet bean [*Mucuna pruriens* (L.) DC. var. *utilis*] is one such species that was long cultivated in tropical regions as a soil-improving crop, a cover crop to control weeds, green manure, and a forage plant (Anaya, 1999; Tarawali et al., 1999; Vargas-Ayala et al., 2000).

Using *in vitro* bioassays and field studies, Fujii et al. (1991) identified secondary chemical agents produced and released by the leaves and roots of *Mucuna*. The main phytotoxic compound encountered was the nonprotein amino acid L-3,4-dihydroxyphenylalanine (L-DOPA), which represented about 1% of the fresh tissues. L-DOPA exudes from *Mucuna* roots to the soil (Furubayashi et al., 2005), and its concentration may reach 1 ppm in water culture solution and 50 ppm in the vicinity of roots (Nishihara et al., 2005). This concentration is enough to affect the growth of neighboring plants by reducing seed germination and suppressing root growth more than shoots of other plant species (Fujii et al., 1991; Fujii, 1999; Nakajima et al., 1999; Nishihara et al., 2004, 2005; Hachinohe et al., 2004). L-DOPA's physiological mechanism in plants, however, is poorly understood. In animals, L-DOPA is a precursor of the neurotransmitter dopamine and the most effective therapeutic agent for the symptomatic relief of Parkinson's disease (Mercuri and Bernardi, 2005). In plants, L-DOPA is a precursor of many alkaloids, catecholamines, flavonoids, melanin, and phenylpropanoids (Hahlbrock and Scheel, 1989).

The phenylpropanoid pathway is an important metabolic pathway as it is involved in the synthesis of phenolic compounds and a wide range of secondary products in plants, including lignin. Phenylalanine ammonia-lyase (PAL) is the first rate-limiting enzyme of the phenylpropanoid pathway leading to the synthesis of the phenolic acids, and later, monolignols (Boerjan et al., 2003). Peroxidase (POD) within the cell wall, in either the free or bound state, is associated with monolignol polymerization and, therefore, in lignin synthesis (Passardi et al., 2005). To date, no reports on the effects of exogenous L-DOPA on soybean roots are available. Because of the important role of lignification in root growth, the question in the current research was whether L-DOPA affects PAL and POD activities and/or phenolic compound and lignin content of soybean roots.

Methods and Materials

General Procedures Soybean [*Glycine max* (L.) Merr. *cv.* BRS-133] seeds, surface-sterilized with 2% sodium hypochlorite for 5 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on three sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10×16 cm) filled with 200 ml of half-strength Hoagland's solution (pH 6.0), without or with 0.1 to 1.0 mM L-DOPA. The container was kept in a growth chamber (25°C, 12-hr light to 12-hr darkness photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). Roots were measured at the beginning and end of the experiments (24 hr). The fresh root weight was determined immediately after incubation, and the dry weight was estimated after oven-drying at 80°C for 24 hr. L-DOPA was purchased from Sigma Chemical Co. (St Louis, MO, USA), and all other reagents used were of the purest grade available or chromatographic grade.

Enzymatic Assays After incubation, all treated or untreated seedling roots were detached, and enzymes were extracted. Phenylalanine ammonia-lyase (PAL) was extracted as described by Ferrarese et al. (2000). Fresh roots (2 g) were ground at 4°C in 0.1 M sodium borate buffer

(pH 8.8). Homogenates were centrifuged (2,200×*g*, 15 min), and the supernatant was used as the enzyme preparation. The reaction mixture (100 μmol sodium borate buffer, pH 8.7, and a suitable amount of enzyme extract in a final volume of 1.5 ml) was incubated at 40°C for 5 min for the PAL activity assay. Fifteen micromoles of L-phenylalanine were added to start the reaction, which was stopped after 1 hr of incubation by the addition of 50 μl of 5 N HCl. Samples were filtered through a 0.45-μm disposable syringe filter and analyzed (20 μl) with a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with an LC-10AD pump, a Rheodine® injector, an SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® GLC-ODS (M) column (150×4.6 mm, 5 μm) was used at room temperature, with an equivalent pre-column (10× 4.6 mm). The mobile phase was methanol to water (70%:30%) with a flow rate of 0.5 ml min^{-1}. Absorption was measured at 275 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). *t*-Cinnamate, the product of PAL, was identified by comparing its retention time with standard values. Parallel controls without L-phenylalanine or with *t*-cinnamate (added as an internal standard in the reaction mixture) were performed as described elsewhere (Ferrarese et al., 2000). PAL activity was expressed as μmol *t*-cinnamate hr^{-1} g^{-1} of fresh weight.

Peroxidase (POD) was extracted from fresh roots (0.5 g) with 67 mM phosphate buffer (5 ml, pH 7.0). The extract was centrifuged (2,200×*g*, 5 min, 4°C), and the supernatant was used to determine the activity of soluble POD. For cell wall-bound POD isolation, the pellet was washed with deionized water until no soluble POD activity was detected in the supernatant. The pellet was then incubated in 1 M NaCl (2 ml, 1 hr, 4°C), and the homogenate was centrifuged (2,200×*g*, 5 min). The supernatant contained the cell wall (ionically)-bound POD. Guaiacol-dependent activities of the soluble and cell wall-bound POD were determined according to Cakmak and Horst (1991), with slight modifications. The reaction mixture (3 ml) contained 25 mM sodium phosphate buffer, pH 6.8, 2.58 mM guaiacol, and 10 mM H_2O_2. The reaction started by adding the enzyme extract into the phosphate buffer. The guaiacol oxidation was followed for 5 min at 470 nm, and enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}) for tetraguaiacol. The blank consisted of a reaction mixture without enzyme extract, and its absorbance was subtracted from the mixture with enzyme extract. POD activities were expressed as μmol tetraguaiacol min^{-1} g^{-1} fresh weight.

Absorption of L-DOPA by Soybean Roots Exogenous L-DOPA-treated or untreated roots (0.1-g dry weight) were put into 5 ml of deionized water in 30 ml polypropylene tubes. Extraction was performed by immersing the tubes into a sonication bath for 5 min (St-Laurent et al., 2002). Extracts were then centrifuged at 2,200×*g* for 2 min and diluted (1:1) in the chromatographic mobile phase containing water to methanol to phosphoric acid (97.5:2:0.1). The solution was filtered through a 0.45-μm disposable syringe filter and analyzed (20 μl) with a Shimadzu® Liquid Chromatograph (Tokyo, Japan), as described earlier. A reversed-phase Shimpack® GLC-ODS (M) column (150×4.6 mm, 5 μm) was used, at room temperature, with an equivalent pre-column (10×4.6 mm). The mobile phase was applied with a flow rate of 0.5 ml min^{-1}. Absorption was measured at 280 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). L-DOPA was identified by comparing its retention time with that of the standard. The L-DOPA concentration in the sample = standard concentration × peak area of the sample / peak area of the standard. L-DOPA absorption was expressed as mg g^{-1} of dry weight.

Phenolic Compound Quantification Dry root (0.25 g) was boiled for 30 min in 5 ml of 2 N HCl. After cooling, the homogenate was filtered through a Whatman® paper, and the filtrate was used to determine the total phenolic compound content. Samples (5 ml) were mixed with 0.75 ml of 1.9 M Na_2CO_3 plus 0.25 ml of Folin-Ciocalteau phenol reagent. This mixture was kept in the dark at room temperature (23–25°C) for 1 hr before its absorption was read at 750 nm (Blum et al., 1991). Ferulic acid was used as the standard. As L-DOPA absorbed by the roots might lead to overestimated results, data obtained by high performance liquid chromatography (HPLC) (described earlier) were subtracted from the total phenolic compound content. Results were expressed as mg total phenolic compound content g^{-1} dry weight.

Lignin Quantification After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 ml, pH 7.0) with a mortar and pestle and transferred into a centrifuge tube (Ferrarese et al., 2002). The pellet was centrifuged (1,400×*g*, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (7 ml); 3× with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 ml); 2× with 1 M NaCl in pH 7.0 buffer (7 ml); 2× with distilled water (7 ml); and 2× with acetone (5 ml). The pellet was dried in an oven (60°C, 24 hr) and cooled in a vacuum desiccator. The dry matter obtained was defined as a protein-free cell wall fraction. Further, all dry protein-free tissue was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 ml of thioglycolic acid plus 6 ml of 2 M HCl) and heated (95°C, 4 hr). After cooling at room temperature, the sample was centrifuged (1,400×*g*, 5 min), and the supernatant was discarded. The pellet contained the complex lignin–thioglycolic acid (LTGA). The pellet was washed 3× with distilled water (7 ml), and the LTGA was extracted by shaking (30°C, 18 hr, 115 oscillations min^{-1}) in 0.5 M NaOH (3 ml). After centrifugation (1,400×*g*, 5 min), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 ml) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 ml). After precipitation (0°C, 4 hr), LTGA was recovered by centrifugation (1,400×*g*, 5 min) and washed 2× with distilled water (7 ml). The pellet was dried at 60°C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Lignin was expressed as mg LTGA g^{-1} dry weight.

Statistical Design The experimental design was completely randomized, and each plot was represented by one glass container with 25 seedlings. Data are expressed as the mean of three to six independent experiments±SE. Significant differences were verified by the one-way analysis of variance with *GraphPad Prism*® package (Version 2.0, GraphPad Software Inc., USA, 1995). Differences between parameters were evaluated by the Dunnett's multiple comparison test, and P values < 0.05 were considered as statistically significant.

Results

To evaluate if L-DOPA had been absorbed by the roots, soybean seedlings were incubated with this compound (0.1 to 1.0 mM) for 24 hr. Fig. 1 shows that L-DOPA was progressively absorbed by the roots. After exogenous 0.1-mM treatment, L-DOPA content determined in

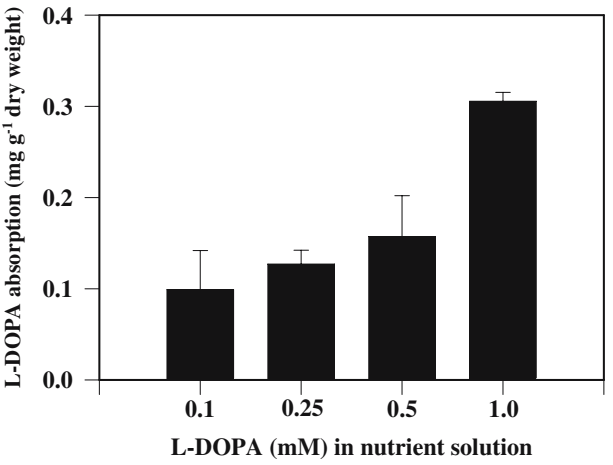


Fig. 1 Absorption of L-DOPA by roots treated exogenously with the allelochemical (0.1 to 1.0 mM) for 24 hr. Mean values±SE from three independent experiments with 25 seedlings for each determination are shown

the roots reached 0.099-mg g^{-1} dry weight, whereas at 1.0 mM, the content amounted to 0.306-mg g^{-1} dry weight.

Root lengths and root fresh and dry weights decreased with increasing concentrations in soybean seedlings grown during short-term exposure (24 hr) in nutrient solution containing L-DOPA (Fig. 2). Mean total root lengths (Fig. 2a) were 20.9, 72.5, and 76.7% less than the control (2.15±0.101 cm) for 0.25, 0.5, and 1.0 mM treatments, respectively. Low concentration (0.1 mM) did not impair root length. Root fresh weight (Fig. 2b) decreased 11.2 and 14.3% at 0.5- and 1.0-mM treatments, respectively, when compared to the control (2.58±0.012 g). A similar trend was also evident in root dry weights (Fig. 2c) determined as 16.1 and 14.9% less than the control (0.174±0.001 g) with 0.5 and 1.0 mM L-DOPA.

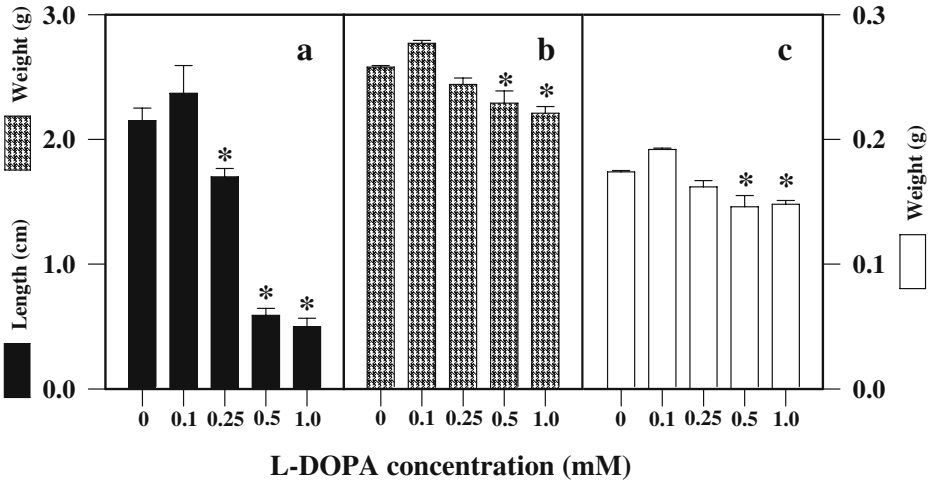


Fig. 2 Effects of L-DOPA on root length (**a**), root fresh (**b**), and dry (**c**) weights of soybean seedlings. Mean values±SE from five independent experiments with 25 seedlings for each determination are shown. *Significant at $P<0.05$ level as compared with the control seedlings

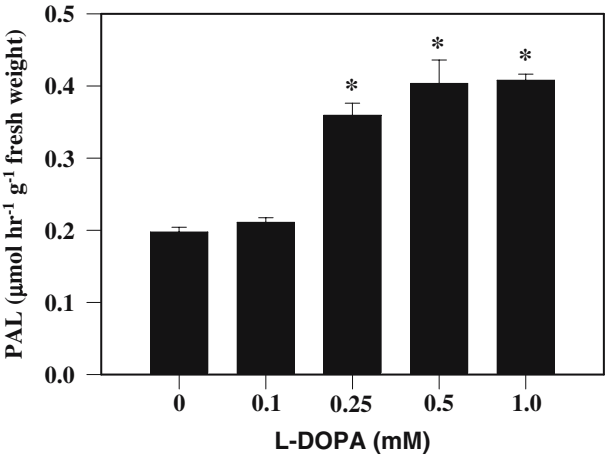


Fig. 3 Effects of L-DOPA on phenylalanine ammonia-lyase (PAL). Mean values±SE from four independent experiments with 25 seedlings for each determination are shown. *Significant at $P<0.05$ level as compared with the control seedlings

No significant differences in the fresh and dry weights of roots exposed to L-DOPA at low concentrations (≤0.25 mM) were recorded.

L-DOPA-affected PAL activities were significantly different from the controls (Fig. 3). The allelochemical increased the enzymatic activities from 82 to 106% after 0.25- to 1.0-mM treatments. No significant change was observed in PAL activity after 0.1 mM L-DOPA exposure. Soluble POD activities (Fig. 4a) increased around 39%, and cell wall-bound POD activities (Fig. 4b) increased around 31% regardless of L-DOPA concentrations, when compared to control conditions.

The phenolic compound content was significantly increased from 86% (at 0.1 mM) to 150% (at 1.0 mM) when compared to untreated roots (Fig. 5). Finally, data revealed that the

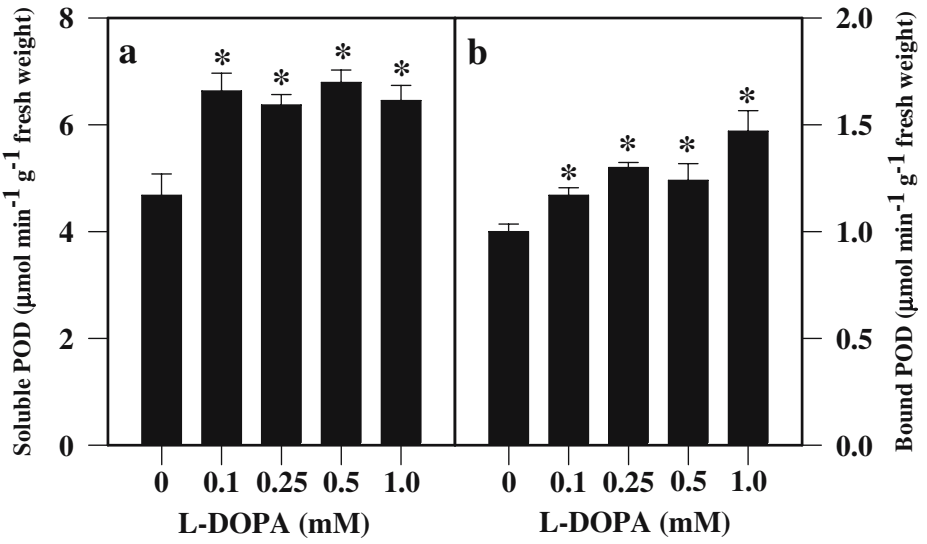


Fig. 4 Effects of L-DOPA on soluble (**a**) and cell wall-bound (**b**) peroxidases (POD). Mean values ± SE from five independent experiments with 25 seedlings for each determination are shown. *Significant at $P<0.05$ level as compared with the control seedlings

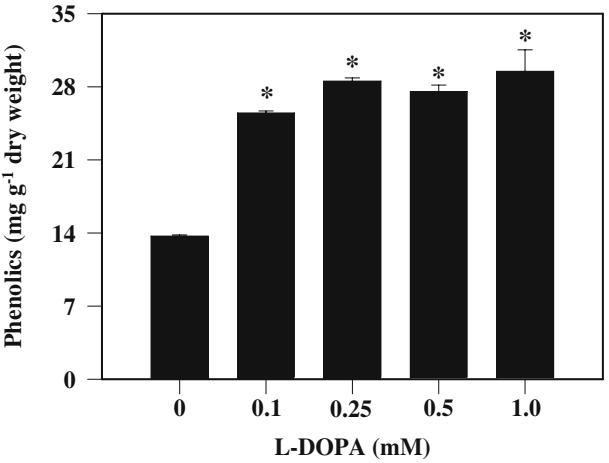


Fig. 5 Effects of L-DOPA on total phenolic compound content. Mean values ± SE from four independent experiments with 25 seedlings for each determination are shown. *Significant at $P<0.05$ level as compared with the control seedlings

lignin content increased around 56% from 0.25- to 1.0-mM L-DOPA, whereas no significant change was recorded at 0.1-mM treatment when compared to untreated roots (Fig. 6).

Discussion

The main finding revealed in the current research is that root growth (length and weight) decreased (Fig. 2) after L-DOPA absorption (Fig. 1), whereas PAL and POD activities and phenolic compound and lignin content increased (Figs. 3 to 6). Another finding is that there is no dose dependency (Dayan et al., 2000). No significant differences among the L-DOPA concentrations (0.25 to 1.0 mM, for example) were recorded for most of the variables analyzed. Therefore, dose-response curves indicating the inhibition threshold for root growth (Fig. 2) or the lowest concentration that completely activates all other variables (Figs. 3 to 6) could not be accurately determined.

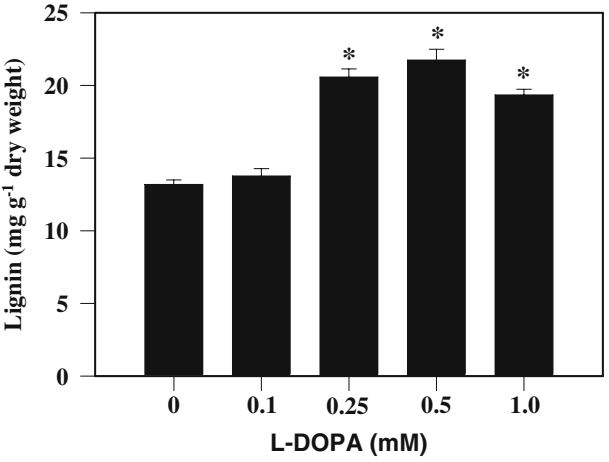


Fig. 6 Effects of L-DOPA on lignin contents. Mean values ± SE from six independent experiments with 25 seedlings for each determination are shown. *Significant at $P<0.05$ level as compared with the control seedlings

Reduction of root growth by L-DOPA was reported in several plant species. Nishihara et al. (2004) used a treatment of 250 μg/ml (about 1.27 mM) and showed that L-DOPA strongly inhibited (<25% of control) root growth of cabbage (*Brassica oleraceae*), komatsuna (*Brassica campestris*), qing gin cai (*Brassica chinensis*), baby blue eyes (*Nemophila menziesii*), watercress (*Nasturtium officinale*), pumpkin (*Cucurbita moschata*), lettuce (*Lactuca sativa*), and cucumber (*Cucumis sativus*). However, tall fescue (*Festuca arundinacea*), perennial ryegrass (*Lolium perenne*), creeping bentgrass (*Agrostis palustris*), red clover (*Trifolium pratense*), milk vetch (*Astragalus sinicus*), hairy vetch (*Vicia villosa*), and corn (*Zea mays*) were less inhibited (>75% of control) and suggested a possible resistance to L-DOPA. The effect of L-DOPA (0.01 or 0.1 mM) on root growth of 32 species was examined at the germination stage (Hachinohe et al., 2004). Root growth was <50% of the control value for 7 of the 19 species treated with 0.01 or 0.1 mM L-DOPA, respectively. Barnyard grass (*Echinochloa crus-galli*) was more tolerant than lettuce, one of the most susceptible species (about 80% inhibition by 0.1 mM L-DOPA). As L-DOPA reduced the soybean root growth and fresh and dry weights (Fig. 2), its susceptibility was indicated, and the amino acid's role as a strong allelochemical was reinforced.

Investigations of the absorption of L-DOPA in plant species are scanty. Using ^{14}C-L-DOPA, Hachinohe et al. (2004) reported that this compound was largely absorbed and metabolized by the roots of barnyard grass and lettuce. The authors verified that metabolites (dopamine, phenylalanine, and tyrosine) derived from ^{14}C-L-DOPA are not directly involved in L-DOPA phytotoxicity. As results reported in the present work indicate that L-DOPA was effectively absorbed by soybean roots (Fig. 1), reduction of root growth by L-DOPA verified after 24-hr treatments (Fig. 2) may be because of this active form in the roots.

The discovery that L-DOPA stimulated lignin production (Fig. 6) is of particular interest. Reduction of seedling roots was associated with premature allelochemical-induced lignification of the cell walls accompanied by increases in phenolic compound content and PAL and POD activities. PAL participates in root growth reduction of maize (Devi and Prasad, 1996), cucumber (Politycka, 1999), and soybean (Herrig et al., 2002; Santos et al., 2004) under stress of cinnamic and benzoic acids derivatives. In these cases, the increase in PAL activity was associated with lignin production. Likewise, higher activities of PAL (Fig. 3) in soybean root tissues were accompanied by an increase in phenolic compound content (Fig. 5) after L-DOPA treatments.

Phenolic compounds play a vital role in plant growth and regulation and also in protecting plants from abiotic and biotic stresses. They are known for their antioxidative properties of scavenging free radicals (Appel, 1993). Phenolic compounds are derived originally by enzymatic deamination of phenylalanine and tyrosine via nitrogen-free skeletons of cinnamate or *p*-coumarate. Once formed, the latter compounds are converted, within the phenylpropanoid metabolism, into a broad range of secondary metabolites such as caffeic, ferulic, and sinapic acids, and eventually, lignin (Boerjan et al., 2003). PAL and POD are involved in this pathway, and the production of phenolic compounds was often observed when levels of phenylpropanoid pathway intermediates or end products are artificially increased. Stress caused by the addition of exogenous allelochemicals (ferulic and vanillic acids) in soybean stimulates the accumulation of phenolic compounds and lignin (Herrig et al., 2002; Santos et al., 2004).

As mentioned earlier, L-DOPA is a precursor of phenylpropanoids. L-DOPA-treated roots of cucumber accumulate nontoxic amino acids (phenylalanine and tyrosine) supposedly because of the oxidation of the allelochemical (Nakajima et al., 1999). In contrast,

dopamine (a product of enzymatic decarboxylation of L-DOPA), tyrosine (the substrate of tyrosine decarboxylase to form dopamine), and phenylalanine (the substrate of PAL in the first reaction of the phenylpropanoid pathway) failed to increase in lettuce roots after exogenous L-DOPA treatments (Hachinohe et al., 2004). In the authors' opinion, these metabolites are not directly involved in the phytotoxicity of L-DOPA as they may be further metabolized into other compounds. It is known that phenylalanine and tyrosine are precursors of phenylpropanoids and lignin (Boerjan et al., 2003). As reported here, soybean is susceptible to L-DOPA (Fig. 2). Although phenylalanine and tyrosine were not monitored in soybean roots treated with L-DOPA, it is reasonable to suppose that the increases in PAL activity (Fig. 3) and phenolic compound content (Fig. 5) may be because of the utilization of these amino acids in the phenylpropanoid pathway.

Increased activities of soluble POD (Fig. 4a) associated with reduced root growth (Fig. 2) induced by L-DOPA are consistent with one of the several functions of this enzyme in plants. Soluble POD mediates the oxidation of L-DOPA to form dopaquinone, and further, melanin (Albrecht and Kohlenbach, 1990; Takahama and Egashira, 1991; Nishihara et al., 2004). Melanin biosynthesis is linked to the production of reactive oxygen species (ROS) or other L-DOPA oxidation products such as semiquinone radicals, quinones, and metal ion-L-DOPA complexes (Kruk et al., 1999; Pattison et al., 2002). In general, ROS and quinones have the potential to interact with many cellular components, triggering stresses in plants, leading to membrane damage, blocking of nutrient uptake and hindering of root growth (Appel, 1993; Einhellig, 1995). Hachinohe and Matsumoto (2005) have recently reported that L-DOPA increases the levels of melanin and thiobarbituric acid-reactive substances (TBARS) in lettuce, which suggests that this action is because of ROS released during melanin synthesis. As the soybean radicle becomes light brown or black after 0.1 and 1.0 mM L-DOPA treatments, respectively, it is reasonable to suppose that the increase in soluble POD activities (Fig. 4a) may be because of oxidation of L-DOPA, and possibly, to melanin formation.

L-DOPA also increased cell wall-bound POD activities (Fig. 4b). One of the noteworthy features of cell wall-bound POD is its association with growth-restricting conditions linked to cell wall stiffening (Passardi et al., 2005). This enzymatic form polymerizes monolignols from phenolic compounds such as ferulic, *p*-coumaric, and caffeic acids. This process requires oxidative coupling and is dependent on H_2O_2. If cell wall-bound POD regulates cell wall stiffening and lignin polymerization (Boerjan et al., 2003), there must be a sufficient supply of H_2O_2. This relatively stable form of ROS may be released during oxidative transformation of L-DOPA into melanin (Kruk et al., 1999). As observed here, increased activities of cell wall-bound POD were associated with a significant decrease in the root length and fresh and dry weights (Fig. 2), and an increase in the lignin content (Fig. 6). Thus, L-DOPA may decrease the root growth because of premature cell wall lignification induced by cell wall-bound POD.

It has become clear from this study that L-DOPA treatment of soybean seedlings results in an increase of PAL activity and increased phenolic compound content. Furthermore, H_2O_2-dependent POD reactions convert phenolic substances into phenoxyl radicals that may produce lignin monomers. These monomers form a complex network that solidifies the plant cell wall and subsequently restricts root growth. According to the results of the current research, it is probable that L-DOPA-induced inhibition in soybean roots may be because of a cell wall stiffening process related to the formation of cross-linking among cell wall polymers and lignin production. If this is the working mode of L-DOPA, the cell wall may be a site for allelopathic action and may indicate how the compound affects the growth of

plant species. One may ask whether these effects are exclusively because of L-DOPA itself or to products of its metabolism. Notwithstanding the fact that L-DOPA was absorbed by roots (Fig. 1), its metabolism was not explored in soybean seedlings. However, Hachinohe and Matsumoto (2005) evaluated the L-DOPA metabolism in other plant species and suggested that its phytotoxicity was because of metabolism and not to its absorption. Therefore, the possibility cannot be discarded that data reported here are results of L-DOPA-induced oxidative stress because of ROS released during its transformation into melanin and/or because of its auto-oxidative breakdown. Further studies are required to elucidate the L-DOPA metabolism in soybean roots and to identify ROS generated during this pathway. This is the challenge of a new study in progress.

Acknowledgement Research was financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). O. Ferrarese-Filho and M.L.L. Ferrarese are research fellows of CNPq. A. R. Soares is the recipient of a CNPq fellowship. The authors kindly thank Aparecida M. D. Ramos and Gisele A. Bubna for their technical assistance.

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J Chem Ecol (2007) 33:277–286
DOI 10.1007/s10886-006-9219-4

Effect of Intraspecific Competition and Substrate Type on Terpene Emissions from Some Mediterranean Plant Species

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Received: 26 July 2006 / Revised: 4 October 2006 / Accepted: 30 October 2006 /
Published online: 29 December 2006
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Abstract Competition is an important factor that has been extensively reported in the Mediterranean area. There is evidence that leaf terpene accumulation may vary between plants growing on calcareous and siliceous soils. In the present study, leaf terpene emissions from potted seedlings of *Pinus halepensis, Cistus albidus*, and *Quercus coccifera*, growing under natural environmental conditions on calcareous and siliceous substrates, were studied by using a bag enclosure method. In both substrates, seedlings were potted alone and in intraspecific competition, to examine the effect of substrate type and that of intraspecific competition on terpene emissions. The results showed that competition favored: (i) overall monoterpene and sesquiterpene emissions from *Q. coccifera*; (ii) overall monoterpene emissions from *P. halepensis*; (iii) overall sesquiterpene emissions from *C. albidus*. Substrate type affected terpene emissions to a limited extent and in a species-specific way. Whereas for *Q. coccifera*, the overall monoterpene emissions and that of Allo-aromadendrene were favored on siliceous substrate, no significant changes were found in emissions from *P. halepensis*. Only the release of AR-curcumene from *C. albidus* was higher on siliceous substrate. We also found high variability in terpene emission composition from the study species, particularly for *P. halepensis* and *Q. coccifera*. These two species released both monoterpenes and sesquiterpenes, instead of monoterpenes only, as shown in previous studies.

Keywords Plant interaction · Soil nutrient availability · Biotic stress factors · BVOC · Sesquiterpenes · Monoterpenes · Secondary metabolism

Introduction

Emission of biogenic volatile organic compounds (BVOC) from leaves is an important mechanism through which plants modify air quality on a global (Guenther et al., 1995), regional

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(Fuentes et al., 2000), and local scale (Simon et al., 2001). In particular, oxidation of terpenes contributes to both ozone (O_3) and secondary organic aerosols (Guenther, 2002). In addition, these natural emissions may increase the life span of greenhouse gases, such as CH_4, which are related to the pattern of global climatic change (Guenther et al., 1995). Consequently, there is interest in understanding which environmental factors affect terpene emissions from plants. This especially concerns Mediterranean regions, as such emissions are directly implicated in O_3 concentration even on a local scale (Simon et al., 2005), and this secondary pollutant exceeds the European tolerated limits several times per year (Millan et al., 1996). Although the scientific community has devoted considerable effort to understand the effect of environmental factors on BVOC in the Mediterranean Region, estimates of these compounds are still uncertain (Staudt et al., 2000). This is partly because of the range of factors controlling these emissions.

With regard to abiotic factors, few studies have addressed the issue of soil nutrient effects on terpene emissions, whereas the effect of climate-related factors is well documented. In contrast to released terpenes, studies dealing with soil nutrient effects on stored or accumulated terpenes within leaves have been more frequent (e.g., Kainulainen et al., 1996; Close et al., 2004). However, those studies were performed on the basis of different fertilizer treatments (Harley et al., 1994; Litvak et al., 1996), and they show inconsistent results. Only a few studies have shown that plant emissions vary on soils of different natural fertility, such as calcareous and siliceous soils. Robles and Garzino, (1998) and Flamini et al. (2004) showed that some terpene content from Mediterranean species varied according to soil type. However, other studies have shown that there is no correlation between terpene content and emissions (Gershenzon et al., 2000). The degree of dependency of terpene emissions on soil type will not necessarily be the same as that found for terpenes stored within leaves.

With regard to biotic interference on emissions, terpenes are currently associated with: (i) attractant compounds that favor pollination or seed dispersal (Caissard et al., 2004); (ii) herbivore and pathogen deterrents (Holopainen, 2004); and (iii) plant to plant communication (Gniazdowska and Bogatek, 2005).

Plant to plant competition is common under natural conditions (Goldberg and Barton, 1992), and one prevalent interaction is competition for limited resources. As soils are often nutrient-poor in Mediterranean regions, the study of competition is of particular interest. Moreover, plant biomass and growth are especially sensitive to competition (Ramseier and Weiner, 2006; Midoko-Iponga et al., 2005), and these factors may explain some BVOC variability among plants of the same species (Batten et al., 1995). Nevertheless, the effect of this factor remains almost wholly unexplored, as only a single study is available on terpene emission response to competition (Peñuelas and Llusià, 1998).

The aim of this work was to examine whether terpene emissions change when plants grow: (i) on calcareous or siliceous substrate, and (ii) alone or in intraspecific competition. We also analyzed whether these two factors interact, as it has been reported that competition for nutrients is more intensive when nutrient availability is high (Vila and Sardans, 1999).

Methods and Materials

Species Studied Terpene emissions from *Pinus halepensis* Mill. (Pinales, Pinaceae), *Cistus albidus* L. (Malvales, Cistaceae), and *Quercus coccifera* L. (Fagales, Fagaceae), which are typical of the Mediterranean area, were studied. The tree species studied, *P. halepensis*, is representative throughout the Mediterranean Basin, where it is widespread (Quézel, 2000). *P. halepensis* (Orshan, 1989) and *C. albidus* (Passama, 1970) grow both on calcareous and siliceous substrates even if they are mainly calcicole species. *Q. coccifera* is rarely found on

siliceous soils (Bornand et al., 1997). All study species form both monospecific and mixed stands in Mediterranean ecosystems. However, they often form dense and abundant monospecific stands in areas that have been previously disturbed by recurrent fire (*Q. coccifera*, Larcheveque et al., 2005; *C. albidus*, Robles, 1998; *P. halepensis*, Barbero et al., 1990).

Experimental Setup The study was performed on 2-yr-old potted seedlings. Seedlings were obtained from plant collections of the nursery garden (Luberon, Lambesc, southern France). For each species, four seedlings were potted alone (four pots, four seedlings) and four other seedlings were potted with another seedling of the same species (in intraspecific conditions) (four pots, four seedlings). In both cases, seedlings were potted on siliceous and on calcareous substrates, thus making a total of 16 seedlings per species. All seedlings were irrigated similarly and regularly to avoid any water stress. Substrates came from natural garrigue sites (of southern France) from the first 25 cm of the soil layer. Soils were sampled in May 2003 and then potting took place over several weeks in summer 2003.

The main physical and chemical properties of calcareous and siliceous substrates were analyzed (Table 1). Calcareous substrate was mainly characterized by higher values of N, Ca^{2+}, TOC (total organic carbon), K^{+}, pH, clay, and silt (Table 1), whereas siliceous substrate showed mainly higher values of Mg^{2+} and sand.

Terpene emission sampling was performed from 15–19 April 2004, from 11:00 to 15:30 (solar time). Experimentation was carried out under natural environmental conditions. During the study period, air temperature varied between 23 and 27°C, PAR (Photosynthetically Active Radiation) ranged from 800 to 1,000 μmol m^{-2} s^{-1}, wind speed was negligible, and air humidity was less than 30%. Weather was typical of the spring season, with alternative cloudy and sunny sky.

Terpene Sampling A semidynamic bag enclosure system, made of Teflon film (FEP), was applied to enclose a single healthy twig per plant. The health of leaves was visually checked. Sun- and shade-exposed leaves, as well as primary and secondary leaves, were considered. The emission rate obtained through this method may be taken as a representation of the mean emission rate. The system consisted of 16 bag enclosures. Each (0.5 l) was designed with two air streams, inlet and outlet.

Table 1 Physical and chemical properties of the calcareous and siliceous substrates studied

Properties	Siliceous Substrate	Calcareous Substrate
pH (H_2O)	6.18±0.20	7.22±0.09
TOC (total organic C) (%)	1.80±0.28	5.96±0.35
Total N (%)	0.084±0.005	0.334±0.005
C/N	20.87±2.05	17.75±0.86
Available P ($mg.kg^{-1}$) (Olsen)	36.14±4.24	32.43±1.12
Total P ($mg.kg^{-1}$)	277.28±34.83	724.57±38.15
K^{+} ($mg.kg^{-1}$)	190.60±20.66	501.70±43.08
Ca^{2+} ($mg.kg^{-1}$)	2052.71±156.47	9768.20±319.81
Mg^{2+} ($mg.kg^{-1}$)	127.78±9.43	102.44±46.12
Sand (% of fine and coarse earth)	74.72±1.91	28.06±3.61
Silt (% of fine and coarse earth)	15.71±3.02	30.56±4.30
Clay (% of fine and coarse earth)	10.1±1.77	22.95±3.21
Texture (Aubert, 1978)	Sandy loam	loam

[a] Values are mean ± SE. N=8.

Before samples were taken, the bag air was renewed, and each bag was continuously flushed with nonpolluted air (Alphagaz, 99.99% purity, Type 1) through a Tygon tube (Tygon® Fuel and lubricant tubing; o.d: 11.2 mm, i.d: 8 mm), especially designed for hydrocarbon transport. Inflowing air (Qe) was measured with a digital mass-flow controller (Aalborg® CFC17, 0–500 ml). Qe was 110±20 ml min^{-1} and was maintained for 15 min, so that the bag air was renewed three times. After air renewal, terpene sampling took place (while inflowing air was maintained). The outgoing terpenes from each bag enclosure were collected on glass sorbent tubes filled with preconditioned Tenax TA (Varian®: 20–35 mesh, 150 mg, 160×5×3 mm), using a pumping system (Edwards®) placed downstream of the Tenax TA. One sorbent tube per bag was used. We previously demonstrated in the laboratory that there were no significant differences between trapped terpene concentrations in four Tenax TA placed simultaneously at different air stream outlets of the same bag enclosure. Outflowing air (Qs) at each bag enclosure was measured with a bubble flow meter (0–280 ml/min, GPE Meterate 314-140/084), placed immediately after each sorbent tube. Flow meters were specially designed to allow connection to a tube, as each bubble flow meter was linked to the pump by means of a reinforced flexible antioxidant PVC tube (i.d. 0.8 mm). Terpene sampling took place for 10 min at Qs=80±30 ml min^{-1}. Constancy of Qs at each bag enclosure was verified every 2 min. Of the 16 bag enclosures constructed, only 14 were directly used to sample terpene emissions; one was always allocated to a blank with no twig in the gas exchange system, and another was used for measurements of air temperature inside the enclosure. After measurements, each sampled twig was cut off and stored in a portable refrigerator at +4°C until being stored at −20°C in the laboratory. Leaves from each twig were separated and lyophilized to measure dry mass (DM).

Terpene Analyses Adsorbed terpenes in Tenax TA were analyzed through thermal desorption by gas chromatography (GC) fitted with a Flame ionization detector (FID) (HP®5890 series II). Before thermal desorption, a preflush phase was run (3 min, 10 ml min^{-1}, 60°C), to allow humidity in Tenax TA to be evacuated. This phase was also necessary to accurately analyze sesquiterpene adsorbed compounds. Then, thermal desorption (Thermal Desorption Cold Trap injector, Varian®, CP4020-TCT model) was carried out through nitrogen carrier gas (10 min, 50 ml min^{-1}, 250°C) and cryogenic concentration in a silica capillary trap, cooled with liquid nitrogen at −100°C. Finally, compounds were separated in the nonpolar chromatographic column (Ultra 2, 50 m× 0.2 mm×0.25 μm).

The identity of terpenes was confirmed by comparison with standards of high purity (Aldrich-Firminich). Calibration of monoterpene and sesquiterpene factor response, used to calculate their concentrations, was performed periodically throughout the sampling period. In a few cases, when standards were not available, peak identification was achieved by injection of previously extracted terpenes from each species, in Tenax TA. The identity of these compounds was determined by means of a GC (HP®6890) coupled with a mass spectrometer (MS) (HP®5973 Network Mass Selective Detector).

Statistical Analyses and Standard Terpene Emission Calculation Statistical treatments were applied to standardized emissions. In the case of *P. halepensis* and *C. albidus*, standardization at 30°C was carried out with the algorithm proposed by Tingey et al. (1980), as temperature is considered to be the main factor controlling emissions from these storing species in the short-term (Llusià and Peñuelas, 2000). As *Q. coccifera* is a nonstoring species, emissions were standardized at 30°C and 1,000 μmol s^{-1} m^{-2} according to the model of Guenther et al. (1995). Temperature and light are currently agreed to be the

main factors controlling monoterpene emissions from nonstoring species (Staudt et al., 2000). Thus, all emission data presented throughout this study represent standard emissions, expressed as mean ± SE.

Two-factor analysis of variance (ANOVAs) was applied to test the effect of both substrate type and intraspecific competition, and possible interacting effects between factors. This statistical test was performed for the overall monoterpene and sesquiterpene emissions, and for the major compounds released from each species. Data were log-transformed, when necessary, to meet normal requirements for ANOVAs analysis. Statistical analyses were performed by using Statistical Graphics® version 4.1.

Results

P. halepensis mainly emits monoterpenes (Table 2). Overall monoterpene emission rate is 3.12±0.75 μg h^{-1} $g_{DM}{}^{-1}$, whereas that of sesquiterpenes is only 0.29±0.04 μg h^{-1} $g_{DM}{}^{-1}$.

Table 2 Terpene emission composition from leaves of *P. halepensis, C. albidus*, and *Q. coccifera*

Monoterpenes	*P. halepensis*	*C. albidus*	*Q. coccifera*	CAS Number	RI
α-thujene	tc	__	__	2867-05-2	919
α-pinene	X	X	X	80-56-8	926
Camphene	X	__	__	79-92-5	944
sabinene	X	X	X	3387-41-5	960
β-pinene	X	X	X	127-91-3	965
β-myrcene	X	X	tc	123-35-3	969
Δ^3-carene	X	tc	tc	13466-78-9	1010
α-terpinene	tc	__	__	99-86-5	1015
p-cymene	X	__	__	99-87-6	1032
Δ-limonene	X	tc	__	138-86-3	1035
β-ocimene	tc	__	__	13877-91-3	1040
γ-terpinene	X	__	__	99-85-4	1060
Δ-terpinene	X	tc	X	586-62-9	1090
Linalool	X	X	X	78-70-6	1100
Camphor	tc	__	__	76-22-2	1159
Borneol	X	tc	tc	507-70-0	1179
α-terpineol	X	tc	tc	98-55-5	1203
Sesquiterpenes					
Copaene	tc	X	X	3856-25-5	1378
β-bourbonene	X	X	__	5208-59-3	1402
β-caryophyllene	X	X	__	87-44-5	1444
Unknonwn (*m/z*=204)	X	X	X	24406-05-1	1473
α-caryophyllene	X	X	X	6753-98-6	1478
Allo-aromadendrene	X	X	X	25246-27-9	1485
AR-curcumene	__	X	tc	644-30-4	1489
α-zingiberene	X	X	__	495-60-3	1499
α-muurolene	X	X	X	10208-80-7	1516
γ-cadinene	__	X	__	39029-41-9	1519
Δ-cadinene	__	tc	__	483-76-1	1523

[a] X: detected compound

[b] __: not detected compound

[c] tc: compound detected in trace amounts

[d] RI: retention index

The major released compounds are α-pinene and Δ^3-carene. Substrate type does not significantly modify either the overall monoterpene and sesquiterpene emissions (ANOVAs, $P>0.05$; Fig. 1) or those of any major compound (ANOVAs, $P>0.05$; Table 3). Whereas substrate type has no effect on terpene emissions from *P. halepensis*, its overall monoterpene emissions (ANOVAs, $P<0.05$; Fig. 1) and emissions of all major monoterpenes, except those of β-pinene (ANOVAs, $P>0.05$; Table 3), increase significantly in conditions of intraspecific competition, irrespective of substrate type. By contrast, the overall sesquiterpene emissions do not change in competition (ANOVAs, $P>0.05$; Fig. 1)

C. albidus is mainly a sesquiterpene emitter (Table 2). Sesquiterpene emission rate is about threefold that of monoterpenes (4.44 ± 1.08 μg h^{-1} $g_{DM}{}^{-1}$). The major sesquiterpenes emitted are α-zingiberene, followed by AR-curcumene and allo-aromadendrene (Table 3). Among the monoterpenes, only α-pinene shows high emission rates. Neither the overall monoterpene, nor the overall sesquiterpene emission rates, show significant changes on calcareous and siliceous substrates (ANOVAs, $P>0.05$; Fig. 1). Only emissions of AR-curcumene rise when seedlings of *C. albidus* grow on siliceous substrate (ANOVAs, $P<0.05$; Table 3). By contrast, emissions from *C. albidus* are markedly dependent on competition, irrespective of substrate type. The overall sesquiterpene emissions (Fig. 1), and those of β-bourbonene and β-caryophyllene, are significantly higher when this species grows in competition (ANOVAs, $P<0.05$; Table 3).

Finally, *Q. coccifera* releases monoterpenes and sesquiterpenes similarly (Table 2). This species shows the lowest emission rate of species examined here (Fig. 1). The major monoterpenes released are α-pinene, followed by β-pinene and sabinene (Table 3). The major sesquiterpenes released are α-caryophyllene and allo-aromadendrene (Table 3). Emission of the overall monoterpenes is significantly higher when *Q. coccifera* grows on siliceous substrate (*ANOVAs*, $P<0.05$; Fig. 1). This is caused by α-pinene, which is the only major monoterpene emitted in higher amounts when seedlings grow on siliceous

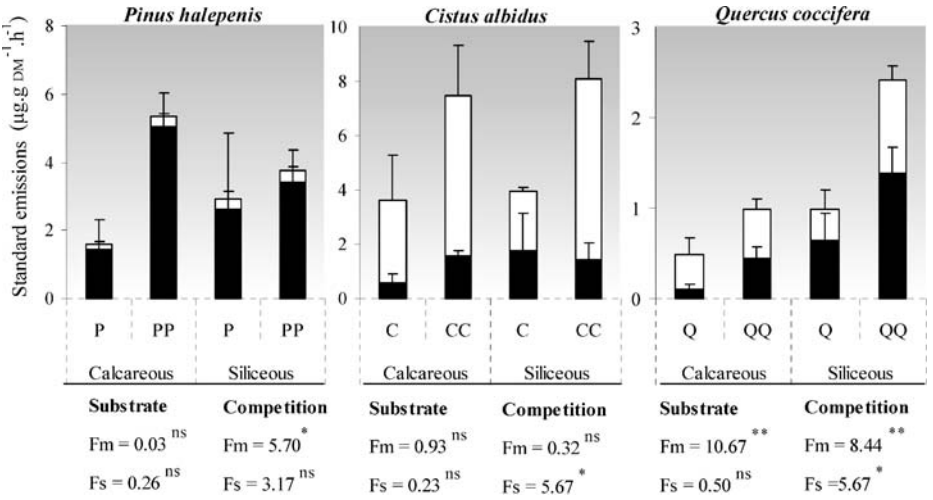


Fig. 1 Monoterpene and sesquiterpene standard emissions from *P. halepensis, C. albidus*, and *Q. coccifera* on calcareous and siliceous substrates, isolated and in intraspecific competition. Values are mean ± SE ($N=4$). Bars indicate the standard error. P, C, Q: isolated seedlings. PP, CC, QQ: seedlings in intraspecific competition. Results of the two-way ANOVAs (substrate and competition) are also shown. *Fm, Fs*: test values for monoterpenes and sesquiterpenes, respectively; ns: not significant at 95% of confidence; *$0.01<P<0.05$; **$0.001<P<0.01$

Table 3 Mean standard emission of each major compound released by leaves of *P. halepensis, C. albidus*, and *Q. coccifera* on calcareous and siliceous substrates, isolated and in intraspecific competition

P. halepensis	Calcareous Substrate	Siliceous SUbstrate	$F_{substrate}$	Isolated	Competition	$F_{competition}$
α-pinene	1.47±0.39	0.88±0.19	1.65^{ns}	0.65±0.32	1.41±0.28	4.80*
β-pinene	0.14±0.02	0.24±0.11	0.03^{ns}	0.12±0.03	0.22±0.08	1.17^{ns}
β-myrcene	0.67±0.29	0.43±0.17	0.40^{ns}	0.14±0.04	0.72±0.23	7.10*
Δ^3-carene	0.75±0.18	1.22±0.33	0.60^{ns}	0.54±0.24	1.14±0.23	5.59*
C. albidus						
α-pinene	0.27±0.11	0.48±0.31	1.48^{ns}	0.10±0.04	0.52±0.28	0.02^{ns}
β-bourbonene	0.28±0.09	0.47±0.12	6.70^{ns}	0.24±0.08	0.46±0.11	4.96*
β-caryophyllene	0.42±0.20	0.45±0.30	0.03^{ns}	0.06±0.02	0.62±0.27	5.32*
allo-aromadendrene	0.69±0.21	0.82±0.18	0.01^{ns}	0.48±0.09	0.91±0.19	0.03^{ns}
AR-curcumene	0.85±0.31	1.02±0.23	6.03*	0.64±0.35	1.08±0.22	0.51^{ns}
α-zingiberene	1.27±0.52	1.14±0.38	0.06^{ns}	0.74±0.33	1.43±0.43	0.08^{ns}
α-muurolene	0.32±0.05	0.64±0.33	0.39^{ns}	0.29±0.07	0.59±0.26	0.01^{ns}
Q. coccifera						
α-pinene	0.10±0.04	0.45±0.30	11.53**	0.20±0.10	0.34±0.10	7.21*
sabinene	0.05±0.03	0.11±0.32	0.45^{ns}	0.04±0.02	0.10±0.04	4.78*
β-pinene	0.09±0.07	0.07±0.32	0.02^{ns}	0.03±0.02	0.10±0.05	0.01^{ns}
α-caryophyllene	0.11±0.02	0.17±0.04	4.60*	0.06±0.03	0.17±0.03	9.83**
allo-aromadendrene	0.13±0.03	0.26±0.06	0.02^{ns}	0.05±0.03	0.14±0.02	6.21*

[a] The effects of substrate type and intraspecific competition on standard emissions of these compounds are tested through two-factor ANOVAs. $F_{substrate}$ and $F_{competition}$ indicate the ANOVA's value for each factor.

Values are mean (n=4)±SE

*0.01<P<0.05

** 0.001<P<0.01

ns = not significant (P>0.05)

substrate (*ANOVAs*, P<0.05; Table 3). The overall sesquiterpene emissions do not significantly change according to substrate type (*ANOVAs*, P>0.05; Fig. 1). Only emissions of α-caryophyllene are higher when seedlings are potted on siliceous substrate (*ANOVAs*, P<0.05; Fig. 1). With regard to competition, the overall monoterpene and sesquiterpene emissions are higher when *Q. coccifera* grows in intraspecific competition (*ANOVAs*, P< 0.05; Fig. 1), both on calcareous and siliceous soils. Emissions of all major compounds, except those of β-pinene, are also higher in competition (*ANOVAs*, P<0.05; Table 3).

Discussion

The aim of the investigation was to extend our understanding of the dependency of terpene emissions on environmental factors. The effect of both substrate type and intraspecific competition, and their potential interaction, were explored on emissions from seedling plants. Despite the recognized importance of competition for different aspects of plant development (e.g., biomass, growth, phenology), little attention has been paid to the impact this biotic factor may have on terpene emissions. The results showed that even if calcareous and siliceous substrates differed in their nutrient resources, competition had a positive effect on terpene emissions among all species irrespective of the substrate type. Thus, this biotic factor had a positive effect on terpene emissions from *P. halepensis* (monoterpenes), *C. albidus*

(sesquiterpenes), and *Q. coccifera* (monoterpenes and sesquiterpenes). Only Peñuelas and Llusià (1998) have previously dealt with the effect of competition on terpene emissions. These authors found similar results to those reported here. They observed that when two seedlings of *P. halepensis* grew together, monoterpene emissions were favored. However, they reported the opposite response for *Q. ilex* where seedlings growing alone showed higher emission rates. Hence, the pattern shown here for *Q. coccifera* in intraspecific competition is different from that reported for *Q. ilex*. Both *Quercus* are long-lived, perennial, and angiosperm species, with a broad edaphic tolerance. Moreover, their emissions have been shown to vary similarly with light and temperature (Niinemets et al., 2002). A possible explanation for the apparent difference in impact of intraspecific competition on terpene emissions between *Q. ilex* and *Q. coccifera* is that Peñuelas and Llusià (1998) added nutrients to substrates during the sampling period, whereas nutrients were not added in our study.

This is the first investigation into plant emissions on calcareous and siliceous substrates, and it shows that, in contrast to intraspecific competition, substrate type affects terpene emissions in a species-specific way. On one hand, monoterpene emissions from *Q. coccifera* were considerably higher on siliceous substrate. On the other, emissions from *P. halepensis* showed similar rates on calcareous and siliceous substrates. Finally, a single major compound released by *C. albidus* showed higher emission rates on siliceous substrate. We think that the higher terpene emissions reported in this study for *Q. coccifera* on siliceous soils is the consequence of stress, as this is a calcicole species that is rarely found on siliceous soils (Bornand et al., 1997).

This study also showed that sesquiterpenes have been neglected, in particular with regard to emissions from *Q. coccifera*, although they may constitute an important fraction of total emitted compounds. The lack of sesquiterpene emission data in previous studies for *Q. coccifera* and *P. halepensis* might, however, be explained by differences in sampling methodology and analysis protocols. Moreover, as sesquiterpenes may alter the tropospheric balance, notably by favoring aerosol formation (Guenther, 2002), their isoprenoids should not be excluded either from terpene emission inventories or from models designed for predicting secondary pollutant concentrations and climate change.

In summary, this study was carried out to contribute to a better understanding of environmental factors that modify the release of terpenes by plants. The results presented could be taken into account: (i) to update terpene emissions from these species in current terpene emission inventories, as sesquiterpenes have not so far been considered; (ii) as input in models commonly used for predicting terpene emissions from plants to obtain more accurate estimations; (iii) to obtain better insight into factors that modify the secondary metabolism of plants. In a broader perspective, it might be of interest to examine whether terpene emissions from these and other Mediterranean species show the same response to the factors analyzed here over the whole seasonal cycle.

Acknowledgments This research was primarily funded by the French Agriculture Minister (DERF), the Environmental agency (ADEME), and PACA region (Presence-Alpes-Côte d'Azur). We thank Sylvie Dupouyet for collaboration in measurement campaigns. We are grateful to Christine Ballini for advice on soil analysis. We are also grateful to Patrice Brahic, the director of the national nursery garden "Aix Les Milles," and Michael Paul for improvements in the English.

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J Chem Ecol (2007) 33:287–296
DOI 10.1007/s10886-006-9235-4

Do Elevated Temperature and CO_2 Generally Have Counteracting Effects on Phenolic Phytochemistry of Boreal Trees?

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Received: 11 October 2006 / Accepted: 27 November 2006 /
Published online: 10 January 2007
© Springer Science + Business Media, LLC 2007

Abstract Global climate change includes concomitant changes in many components of the abiotic flux necessary for plant life. In this paper, we investigate the combined effects of elevated CO_2 (720 ppm) and temperature (+2 K) on the phytochemistry of three deciduous tree species. The analysis revealed that elevated CO_2 generally stimulated increased carbon partitioning to various classes of phenolic compounds, whereas an increase in temperature had the opposite effect. The combined effects of both elevated CO_2 and temperature were additive, i.e., canceling one another's individual effects. Obviously, the effects of global climate change on leaf chemistry must simultaneously consider both temperature and CO_2. If these results are generally applicable, then the counteracting effect of the temperature is likely to play a major role in alpine, boreal, and arctic zones in determining the balance between populations of plants and herbivores.

Keywords Climate change · Secondary compounds · Metabolism · *Betula* · *Salix* · Phenolics

Introduction

Global climate change, considered as an increase in atmospheric CO_2 and temperature, can influence interactions between plants and their herbivores by changing the quality of plants as food by making them more stoichiometrically unbalanced for their herbivores (Loladze 2002; Sterner and Elser, 2002). For example, a review of the literature on CO_2 elevation by Bezemer and Jones (1998) revealed that leaf nitrogen concentration decreased on average

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by 15%, whereas carbohydrates and plant phenolics increased by 47% and 31%, respectively. Zvereva and Kozlov (2006), in their metaanalysis of the consequences of the simultaneous elevation of CO_2 and temperature on plant traits, made similar conclusions about CO_2 effects, but concluded that temperature alone typically had opposite effects, whereas temperature and CO_2 combined had negligible effects. Both reviews concluded that insects from different feeding guilds generally responded to CO_2-mediated changes in host-plant quality by compensating for decreased nitrogen levels and increased phenolic levels in the plant tissues by increasing their food consumption, but suffered lowered food conversion efficiencies, and longer larval development.

General circulation models (GCM) indicate that, especially in arctic, alpine, and boreal zones, elevated temperatures will accompany rising levels of CO_2 (Prentice et al., 1992; IPCC 1996). In these geographic zones, the effects of elevated temperature may be especially important because the growth of most plants is typically temperature-limited. Although the effects of elevated atmospheric CO_2 on plant physiological processes has long been studied, the combined effects of elevated temperature and CO_2 are only poorly known. The growth-differentiation balance model of plant development suggests elevated CO_2 may generally increase carbon partitioning to secondary metabolites like phenyl propanoids because it typically increases plant source strength (rates of photosynthate accumulation) more than sink strength (Herms and Mattson, 1992; Mattson et al., 2005). However, the effects of elevated temperature are much less predictable because it is not fully obvious how that will simultaneously influence both source and sink processes and thus the crucial source-sink carbon balance over plant development.

Temperature increases generally raise the rates of biochemical reactions, but such reactions also depend on the supplies of available substrates and enzymes to sustain them. If plant sink (growth) processes are more sensitive to ameliorative changes in temperature than is photosynthesis (Mattson and Haack, 1987a,b; Herms and Mattson, 1992), then the plant's source-sink balance may generally decline with rising temperatures because sink demands will outpace source supplies. This could lead to reduced carbon partitioning to facultative secondary metabolism. Although this reasoning is obviously elementary, as a starting point it suggests that under favorable growing conditions (i.e., adequate nutrients and water), the effects of elevated CO_2 and temperature might be counterbalancing, as was evident in the Zvereva and Kozlov (2006) metaanalysis. However, metaanalysis indicates only pattern or a trend combined from completely separate studies and tells very little about the metabolic processes lying behind the phenomena. In this study, we go a layer deeper to understand better those partitioning processes affected by elevated CO_2 and temperature treatments by using a dataset coming from one controlled experimental system (Kellomäki et al., 2000). Here, we use graphic vector analysis (GVA), which allows us to analyze if the target phytochemical response is associated with changes in total dry weight, or target chemical content, or is some combination of these two partitioning alternatives.

We argue that to predict the effects of global climate-change-mediated responses in phytochemistry and plant–herbivore relationships, the CO_2 × temperature interaction must be more fully considered.

Methods and Materials

Analytical Procedures We employed graphic vector analysis (GVA) for representing and interpreting the fundamental changes in the plants' growth and phytochemical components

in response to different treatments (Timmer and Stone, 1978; Haase and Rose, 1995; Koricheva, 1999). This method, although laborious, has been proposed as an alternative to simply examining phytochemical concentrations to understand environmental effects on carbon partitioning to the synthesis of the target compounds (Koricheva, 1999). If GVA is more insightful than simply evaluating changes in concentrations of target compounds, it is because GVA also requires measuring and evaluating total plant (or organ) growth that is necessary to construct the axes. GVA is, in fact, three-dimensional plotting of target compound concentration (Y axis), total plant (organ) content (X axis), and total plant (organ) size (Z axis). Instead of using raw values, the data are converted to their relative values, using the control as the standard in the following manner:

$$(elevated\ value - control\ value) \times control\ value^{-1}$$

Total relative dry weight growth of a plant is the inverse of the slope of the aforementioned plot of Y vs. X variables, and all such plotted plant-relative dry weights will lie on diagonal lines corresponding to the Z axis (Fig. 1.). If the goal is to measure and evaluate treatment effects on plant carbon partitioning to a target phytochemical compound, there are two basic responses, either increased or decreased relative partitioning, as indicated on the Y axis by relative concentrations being greater or less than 1, respectively. If, however, one also considers how treatments affect relative plant organ size (presented here as specific leaf weight) in addition to partitioning to the target compound, there are eight possible outcomes (Fig. 1, Table 1): increased relative synthesis and a) decreased or b) increased specific leaf weight (*i.e.*, gm^{-2}); decreased synthesis and c) decreased or d) increased specific leaf weight; no change in synthesis and either e) decreased or f) increased specific leaf weight; and finally, g) increase or h) decrease in synthesis, but no change in specific leaf weight.

The use of relative values allows one to present the results of several plant species, and treatments on the same graph. Each point represents the relative mean value of each plant species for each compound in question. We did not test the data statistically, because that was already done in the studies from which these data were extracted (Lavola and

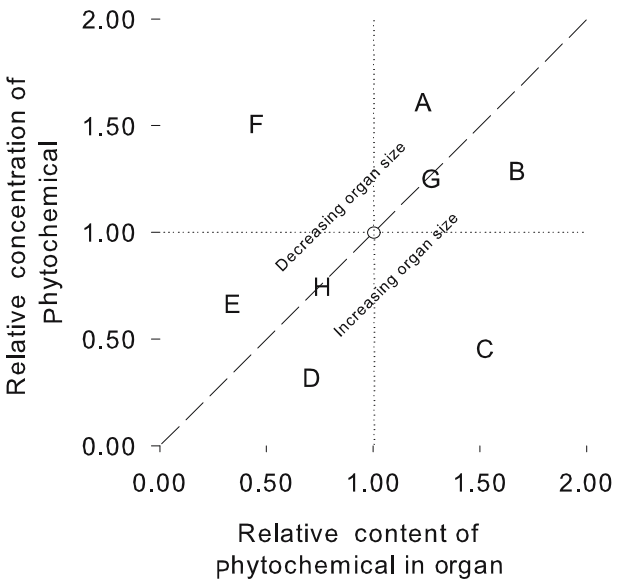


Fig. 1 Interpretation of directional shifts in chemical concentration, chemical content, and specific leaf weight. Modified from Timmer and Stone (1978), Haase and Rose (1995), and Koricheva (1999). Dotted lines represent reference lines for no change in phytochemical character in question. Dashed line indicates relative plant unit biomass, reference point (control sample) has values (1, 1, 1). Letters refer to quadrants in Table 1

Table 1 Interpretations of quadrants in Fig. 1

Graph section	Total dry weight response	Concentration response	Chemical content response	Interpretation: phytochemical synthesis rate relative to growth rate, and overall growth response
A	−	+	+	>relative synthesis/<organ size
B	+	+	+	>relative syntheisis/>organ size
C	+	−	+	<relative synthesis/>organ size
D	+	−	−	<relative synthesis/>organ size
E	−	−	−	<relative synthesis/<organ size
F	−	+	−	>relative synthesis/<organ size
G	0	+	+	>relative synthesis
H	0	−	−	<relative synthesis

Julkunen-Tiitto, 1994; Lavola et al., 1998; Kuokkanen et al., 2001, 2003; Veteli et al., 2002; Veteli 2003; Veteli, unpublished). The direction and magnitude of the vectors from reference or control (1, 1, 1) are used to interpret the effects of the treatments (Fig. 1, Table 1). There are four quadrants in a vector diagram with the diagonal (Z axis = 1) bisecting the upper right and lower left. All vector points to the left of the dashed diagonal are cases where the treatment plants are smaller than control, and all vector points to right of the diagonal are cases where treatment plants are larger. All changes in phytochemical concentrations (Y axis) are the result of shifts in the relative rates of synthesis of both phytochemical content and plant biomass. We avoid the GVA parlance of "diluting" and "concentrating," etc. with respect to the target compound(s) because we believe that it is misleading and inappropriate. Growing plants are continuously adjusting their carbon partitioning based on their unfolding developmental programs, source-sink balances, environmental conditions, and stochastic events. This implies that a target compound is simply being diluted or concentrated in response to treatments and environments, and it is misleading because it suggests a passive process. Instead, carbon partitioning alternatives are of physiological and ecological import to the plant. Having a substantial capacity for changing carbon partitioning at a given time and circumstance is crucial to plant fitness.

Treatments and Studies Included The treatment conditions used in these studies were: (i) control CO_2 and temperature, (ii) elevated CO_2 and control temperature, (iii) control CO_2 and elevated temperature, and (iv) elevated CO_2 and temperature. The mean control and elevated CO_2 concentrations were 360 and 720 ppm, respectively. The elevated temperature was obtained by raising the ambient air temperature by 2 K, on average, from that of the control chambers to correspond to the climate warming scenarios predicted after a doubling of the atmospheric CO_2 concentrations (IPCC 1996). Treatments were done at the Mekrijärvi Research Station, University of Joensuu (62°47′N, 30°58′E, and 145 m a.s.l.), eastern Finland. Sixteen closed-top climate chambers were divided in four CO_2 and temperature treatments (as above), with four replicates in each (Kellomäki and Wang, 1998). Outdoor climate chamber studies (Kuokkanen et al., 2001, 2003; Veteli et al., 2002; Veteli 2003; Veteli, unpublished) and results from indoor chamber studies in which the CO_2 levels were as above (Lavola and Julkunen-Tiitto, 1994; Lavola et al., 1998) were included in the vector analysis.

The tree species used in these experiments (*Betula pubescens*, *B. pendula*, and *Salix myrsinifolia*) are species that are widely distributed and common in boreal zone. Their responses to various environmental factors have been extensively studied and, therefore,

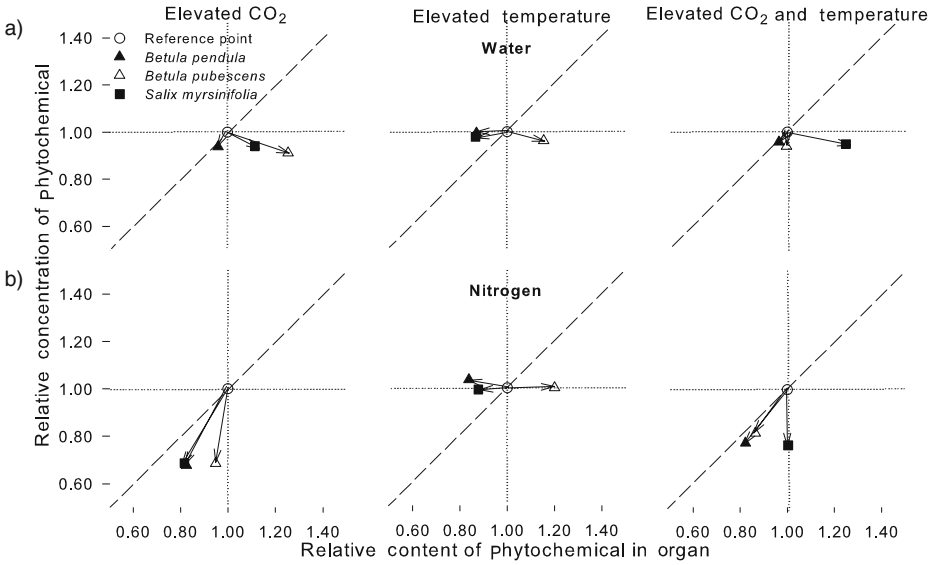


Fig. 2 Directional shifts in A) water and B) nitrogen in relation to climate change factors. The first figure in the column indicates the effect of elevated CO_2, second the elevated temperature, and the third their interaction

provide a fruitful basis for this study. Sampling and chemical analysis are described in detail by Lavola and Julkunen-Tiitto (1994); Lavola et al. (1998); Kuokkanen et al. (2001), (2003); Veteli et al. (2002); and Veteli (2003).

Results

Graphic vector analysis (GVA) shows that specific leaf mass responded rather consistently to treatments. CO_2 enrichment by itself resulted in heavier leaves per area unit on all three tree species (Figs. 2, 3, 4, and 5, first figure in a row). In contrast, elevated temperature by

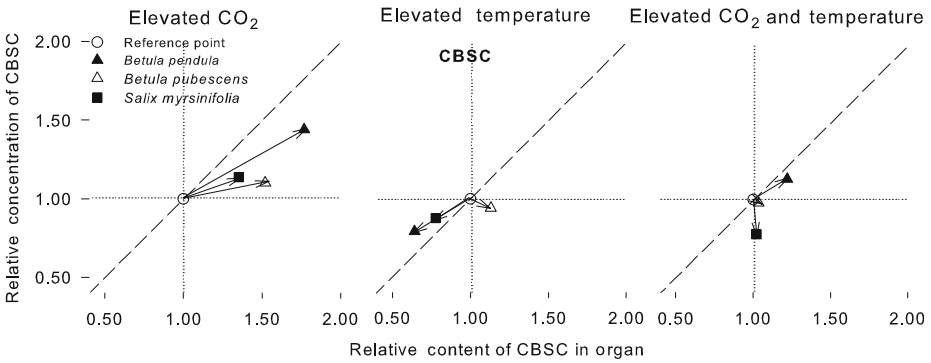


Fig. 3 Directional shifts in detected carbon-based secondary compounds in relation to climate change factors. The first figure in a column indicates the effect of elevated CO_2, second the elevated temperature, and the third their interaction

itself reduced specific leaf weights on two species, but increased that of *Betula pubescens* (Figs. 2, 3, 4, and 5, second figure in a row) The combination of elevated CO_2 and temperature resulted in no net change in specific leaf weight, except for *Salix* where it increased, as if CO_2 overwhelmed the temperature effect (Figs. 2, 3, 4, and 5, third figure in a row).

With respect to foliar chemicals, all graphs generally suggest that there is a counteracting effect between elevated CO_2 and temperature, although the effect is weakest for water and N (Figs. 2, 3, 4, and 5). In the case of water, elevated CO_2 generally increased specific leaf weights but not water uptake, and water concentrations fell. Elevated temperature had negligible effect on water concentrations, whereas the combined effect of CO_2 and temperature caused all plants to have slight decreases in water concentrations, although no declines or even increases in total leaf water content (Fig. 2a). In the case of N, elevated CO_2 generally reduced assumed N uptake and N concentration in all three species (Fig. 2b). Elevated temperature, however, had no effect on foliar N concentrations, although total N content both increased and decreased (Fig. 2b). On the other hand, the combination of CO_2 and temperature caused reduced N uptake and concentrations (Fig. 2b).

With respect to phenolic secondary metabolites, elevated CO_2 generally elicited increased synthesis of detected total carbon-based secondary compounds (CBSC) and thus increased concentrations (Fig. 3). The pattern was the same for most of the component synthetic groups such as phenolic acids, salicylates, flavonoids, and condensed tannins (Figs. 4 and 5). On the other hand, elevated temperature generally elicited diminished synthesis and concentration of total CBSC, and most of the component molecular groups. Tannins were an exception because they were diminished in only *B. pendula*. The combined treatments of CO_2 and temperature elicited steady states or small change in concentrations

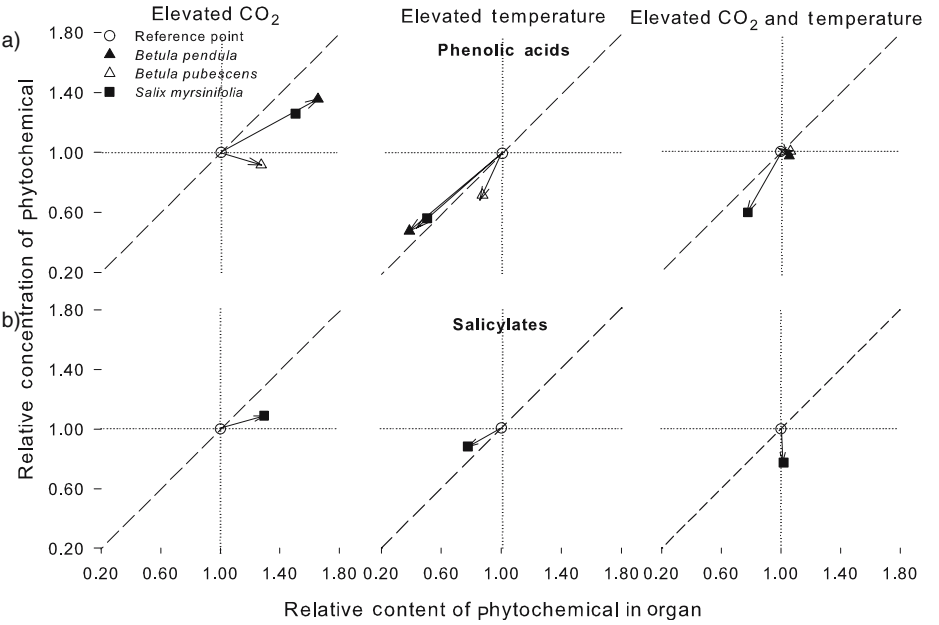


Fig. 4 Directional shifts in A) phenolic acids and B) salicylates in relation to climate change factors. The first figure in a column indicates the effect of elevated CO_2, second the elevated temperature, and the third their interaction

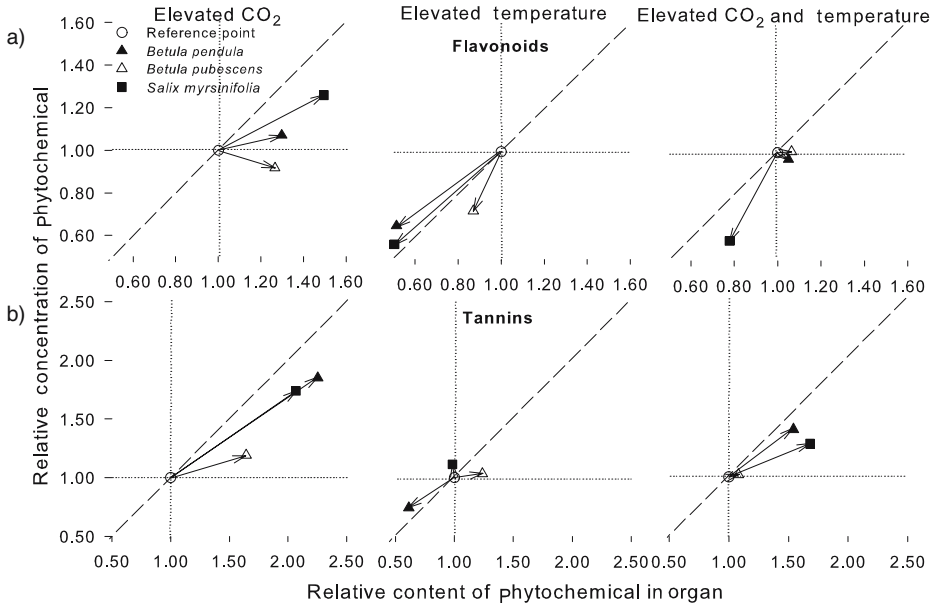


Fig. 5 Directional shifts in A) flavonoids and B) tannins in relation to climate change factors. Notify different scaling in figures A) and B). The first figure in a column indicates the effect of elevated CO_2, second the elevated temperature, and the third their interaction

of secondary compounds in most instances for the two *Betula* species. However, *S. myrsinifolia* was much more responsive. Elevated CO_2 and temperature generally caused substantially reduced concentrations of most phenolics (Figs. 3, 4, and 5a), except for tannins which increased, as was also true for *B. pendula* (Fig. 5b). The data suggest that heightened temperatures generally reduced the magnitudes of the changes that were elicited by elevated CO_2 (Fig. 5b).

Discussion

We showed that the interactions between elevated CO_2 and temperature are typically additive and generally cancel one another. Generally, elevated CO_2 increased the accumulation of total CBSC and specific carbon-based secondary compounds as has been proven in previous studies (Lincoln et al., 1993; Bezemer and Jones, 1998; Hunter, 2001; Zvereva and Kozlov, 2006). However, the climate changes predicted also include rising temperatures, and in this study, changes of only 2 K eliminated the effect of elevated CO_2 on analyzed carbon-based secondary compounds within the leaves. It could be assumed that CO_2 enhancement either increased the synthesis or decreased the turnover rate, and that temperature elevation caused the opposite. For almost all of the plant species previously studied, the carbohydrate concentrations of leaves have been reported to rise by an average of 47% in response to elevated CO_2 (Bezemer and Jones, 1998). In the present study, the carbohydrate concentrations were not measured, but specific leaf weights increased under enriched CO_2, which may be a result of starch accumulating in the leaves. The effect was present also in the combination of the treatments, although not so intense. Starch may or

may not enhance the herbivore's ability to digest leaves, whereas structural carbohydrates (cellulose, hemicellulose), as well as lignin, will very likely interfere with feeding and digestion, especially that of small larvae (Lincoln et al., 1993; Reavey, 1993).

The accumulation of nonstructural carbohydrates under elevated CO_2 in leaves has been reported in many studies (e.g., Johnson and Lincoln, 1991; Lindroth et al., 1993; Hall et al., 2005), but this alone does not explain the reduction in nitrogen concentration observed in this study because total leaf N content also declined. The higher C/N ratio implies perhaps a surfeit of C during leaf ontogeny, thereby allowing more partitioning to facultative synthesis of secondary metabolites. Likewise, both N concentrations and total leaf N were lower in the foliage from the combined treatments, although leaf size was unaffected for the two *Betula* species, but larger for *S. myrsinifolia*. Their C/N ratios were lower than in the CO_2 treatment but higher than in the elevated temperature treatments that were the same as control. Because insect herbivores tend to be nitrogen-limited (Mattson, 1980), possible reductions in nitrogen concentration may substantially reduce insect performance. Thus, it could be expected that the consequences of reduced nitrogen will also prevail in the boreal zone after modest temperature elevation, possibly having a reducing effect on herbivory (see, e.g., Hall et al., 2005). In total, however, the positive effect of temperature may have an ameliorating or even enhancing effect on the growth rates of insects (e.g., Lindroth et al., 1997; Holopainen and Kainulainen, 2004).

Our results here and earlier suggest that elevated CO_2 and temperature can enhance the growth of plants (Kuokkanen et al., 2001, 2004; Veteli et al., 2002; Veteli 2003) while lowering the levels of nitrogen and water in leaf tissue. The effects of elevated CO_2 on levels of CBSC are quite variable depending on the species and compound in question (see, e.g., Agrell et al., 2004; Hall et al., 2005), whereas temperature elevation tends to lower CBSM levels in these species, which is also shown as a general trend in Zvereva and Kozlov's (2006) study. These differing reactions are probably caused by different source–sink interactions: if enhanced plant growth demand is not more than matched by enhanced photosynthesis, there may be a subsequent diminution in available carbon for facultative secondary metabolism. The effect of rising temperature may be similar to the nonlinear effect of fertilization on CBSCs (Herms and Mattson, 1992; Herms, 2002). Some insect species feeding on these plants have increased consumption and reduced growth (e.g., Bezemer and Jones, 1998; Veteli et al., 2002; Zvereva and Kozlov, 2006), and, therefore, may be subjected to natural enemies for a longer time under elevated CO_2. Higher temperatures, on the other hand, may directly compensate for some of the negative effects of increased CO_2; increasing temperatures affect the distribution of some species by shifting ranges poleward and altitudinally upward (e.g., Parmesan et al., 1999; Battisti et al., 2005). The reason for these observed species range shifts can be a result of reducing extreme winter temperatures, increasing thermal sum, or subsequent changes in properties of the host plants.

Hence, we argue that in alpine, boreal, and arctic areas the effect of the rising temperature in addition to levels of atmospheric CO_2 should be taken into account in making any predictions about the effects of climate change on phytochemistry and plant–herbivore–natural enemy interactions (Mattson and Haack 1987a,b). The GCM predicts that in these areas the most important factor of climate change is temperature elevation. We conclude that when studying the effects of climate change we must consider both the effects of enriched CO_2 and elevated temperature. This is especially true in alpine, boreal, and arctic environments where temperature is often the most important factor limiting growth.

Acknowledgments We thank Dr. Marja-Leena Laitinen for valuable hints. We are also grateful to Dr. John Derome for checking the language of this article. This study was supported by the Academy of Finland (Finnish Centre of Excellence Program for Forest Ecology and Management 2000–2005, project no. 64308 and 51997).

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J Chem Ecol (2007) 33:297–309
DOI 10.1007/s10886-006-9214-9

Scopolamine in *Brugmansia Suaveolens* (Solanaceae): Defense, Allocation, Costs, and Induced Response

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Received: 29 May 2006 / Revised: 26 September 2006 / Accepted: 12 October 2006 /
Published online: 29 December 2006
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Abstract *Brugmansia suaveolens* (Solanaceae) contains tropane alkaloids (TAs), which can act as chemical defenses. Selective pressures might modulate the allocation of alkaloids within the plant, as postulated by optimal-defense theory. By tracing scopolamine, the most abundant TA in this species, we found that scopolamine in an artificial diet, in concentrations similar to those in leaves of *B. suaveolens*, increased mortality and prolonged developmental time of the larvae of the generalist noctuid moth *Spodoptera frugiperda*. A diet of undamaged leaves of *B. suaveolens* also showed a large negative effect on the growth of larvae of *S. frugiperda* compared to a diet of leaves of *Ricinus communis*, a species that did not have negative effects on this moth; more valuable plant parts, such as young leaves, flowers, and unripe fruits with seeds, have higher scopolamine concentrations than other tissues; leaves of *B. suaveolens* increase their content of scopolamine after artificial damage. The highest induction was found 24 hr after the damage, and after that, scopolamine content decreased to constitutive levels. This increase represented a cost, because in another experiment, a treatment with methyl jasmonate, an elicitor hormone, increased scopolamine production 9.5-fold and decreased leaf growth 2.3-fold; a diet of artificially damaged leaves of *B. suaveolens* showed a negative effect on the growth of larvae of *S. furgiperda* compared to undamaged leaves, suggesting that damage by herbivores induces resistance. Our data are in line with the optimal-defense theory, but experiments in the field with herbivores that share an evolutionary history with *B. suaveolens* must be undertaken to understand the dynamics of TA allocation in response to herbivory.

Keywords *Brugmansia suaveolens* · Costs of induction · Herbivory · Induced resistance · Induced responses · Artificial damage · Methyl jasmonate · Optimal-defense theory · Scopolamine · Solanaceae · Tropane alkaloids

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Introduction

Plant defenses are costly, and optimal-defense theory, originally outlined by McKey (1974) and later elaborated by Rhoades (1979), predicts that defenses should be preferentially allocated to more valuable plant parts, resulting in a spatial and temporal allocation. Changes in resistance to herbivores of plants following damage, induced responses, are a saving–cost strategy, where the responses and response-associated costs are increased only when the benefit can be realized, e.g., in the presence of the damage (Zangerl and Bazzaz, 1992; Karban and Baldwin, 1997). These responses can be morphological (Young, 1987) or chemical (Zangerl and Bazzaz, 1992; Karban and Baldwin, 1997, and references therein). The latter are generally due to an increase in protease inhibitors or compounds of secondary metabolism. Green and Ryan (1972) made pioneer studies on chemical induced responses, showing that simulated herbivory increases the content of protease inhibitors in leaves of young potato and tomato. Induced responses are also associated with biotic responses of the third throphic level, such as ants (Agrawal, 1998; Romero and Izzo, 2004). The last three decades have shown an increase in studies focusing on chemical induced responses by herbivory, reporting a broad spectrum of secondary metabolites that are inducible (see Karban and Baldwin, 1997, and references therein). Few studies have reported on the induction of tropane alkaloids (TAs) in plants. Andries, cited in Rhoades (1979), found an increase in TA content in *Datura* sp. after nutrient stress. Khan and Harborne (1990) showed, after artificial damage or herbivory by mollusks, that leaves of *Atropa acuminata* (Solanaceae) increased the TA content by 160%. However, Shonle and Bergelson (2000) did not find an induction of these compounds in insect-damaged *Datura stramonium* leaves. TAs are reported to be effective against many herbivorous insects (Hsiao and Fraenkel, 1968; Krug and Proksch, 1993; Wink, 1993; Shonle, 1999). Hyoscyamine, scopolamine, and other esters of tropine constitute a distinctive group of secondary metabolites of the Solanaceae (Griffin and Lin, 2000). They have been found in the subfamilies Schizanthoideae, Solanoideae, and Nicotianoideae, and are especially abundant in certain tribes, e.g., Datureae, Hyoscyameae, and Mandragoreae. In Physaleae, Solaneae, and Solandreae, only a limited number of taxa produce these alkaloids, which are completely absent in other tribes and subfamilies (Wink, 2003).

Brugmansia suaveolens (Humb. & Bonpl. Ex Willd.) Bercht. & J. Presl. (Solanaceae: Solanoideae: Datureae) produces TAs (Freitas et al., 1996). This plant is a small tree native to South America, 2–5 m high, with leaves ovate to narrowly elliptic; large, 20–30-cm-long white flowers; and 10–15-cm-long fusiform fruits. The genus is closely related to *Datura* (Olmstead and Palmer, 1992; Griffin and Lin, 2000; Wink, 2003). In the field, *B. suaveolens* has been reported to be used as host plant by larvae of the specialist ithomiine butterflies *Placidula euryanassa* (=*Placidina euryanassa*, Lamas, 1999) and *Miraleria cymothoe* (Freitas, 1993; Freitas et al., 1996).

In the present work, we investigated whether chemical defense strategies of *B. suaveolens*, involving TAs, matched optimal-defense theory (McKey, 1974, 1979; Rhoades, 1979). We predicted that (1) TAs defend *B. suaveolens* against herbivores, (2) TAs have differential distributions in plant organs according to value for the plant in each phenological phase, (3) TAs are costly, and (4) TAs increase when the plants are subject to herbivore attack. To test these predictions, we performed experiments to verify the effect of scopolamine on the performance of a model of generalist insect herbivore; we analyzed the amount of the TA scopolamine in different plant organs in each phenological phase; we artificially damaged leaves of *B. suaveolens* and verified changes of scopolamine content; we tested whether artificially damaged leaves decreased the performance of a generalist

herbivore; and we verified whether an increase in scopolamine content, thorough elicitation by the phytohormone methyl jasmonate, represented a metabolic cost to *B. suaveolens*. We analyzed scopolamine as a representative TA of *B. suaveolens* because this alkaloid is the major one found in *B. suaveolens* (Freitas et al., 1996), and, biosynthetically, scopolamine is one of the more derived TAs (Croteau et al., 2000). In an escalating arms-race scenario between plants and herbivores, the more derived compound could be expected to be more active against herbivory (Berenbaum, 1983).

Methods and Materials

Plant material Individuals of *B. suaveolens* were collected in semidecidous mesophyll forest, along stream edges in the Serra do Japi, Jundiaí, São Paulo State, Brazil (from 23° 13′21.9″S, 46°58′14.0″W to 23°13′15.1″S, 46°58′5.2″W). A voucher specimen was deposited at the Herbarium of the Institute of Biology (UEC 35387) and at the Herbarium of the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (CPQBA 171). Stem cuttings (several clones of each individual) of 10 different individuals of *B. suaveolens* were rooted for 20 d in flasks containing 0.1% of benomyl solution inside an acclimatized chamber at 25±2°C with a light intensity of 40 μmol/m^2s and a 12-hr photoperiod. Rooted cuttings were transferred to 2-l pots containing a mixture of soil and sand (3:1). Each pot was supplied once with 100-ml full Hoaglands nutrient solution (Hoaglands and Arnon, 1950) to enhance root production and to help in establishment of plants in the pots.

Herbivores Larvae of first instars of the polyphagous noctuid moth *Spodoptera frugiperda* were obtained from Bug Biological Agents, Piracicaba, São Paulo, Brazil (http://www.bugbrasil.com.br/eng/index2.htm), and maintained in an acclimatized chamber (L12:D12, 25±1°C, and about 70% RH). Larvae were reared individually in plastic pots, 4 cm in diam, 5 cm high, on artificial wheat germ diets (Ferraz, 1991).

Performance of a generalist herbivore insect on scopolamine diet To verify negative effects of scopolamine on the performance of generalist herbivore insects, we use as an herbivore model the polyphagous pest insect *S. frugiperda*. Although this generalist noctuid does not share its evolutionary history with *B. suaveolens*, this species or related ones are commonly used as models for generalist insect herbivores to make predictions (e.g., Blau et al., 1978; Miller and Feeny, 1983; Krug and Proksch, 1993; Bultman and Bell, 2003; Dyer et al., 2003; Macel et al., 2005). Second instars of *S. frugiperda* were maintained in an acclimatized chamber (L12:D12, 25±1°C, and about 70% RH), and were reared individually in individual plastic pots, 4 cm in diam, 5 cm high, on artificial wheat germ diets containing the treatments 0, 0.05, 0.25, 0.5, and 2.5% dry weight of commercial scopolamine during their entire development. Before use, scopolamine hydrobromide trihydrate Across™ was basified and extracted ×3 with $CHCl_3$, yielding the free-base scopolamine. We used free-base scopolamine because the form in which this compound occurs in plants containing TAs is controversial. Choi et al. (1999) suggested that both the salt form and free base were present in *Scopolia japonica*. In a recent report of TAs in seeds and leaves of *D. stramonium* var. *tatula*, Mroczek et al. (2006) showed that scopolamine and hyoscyamine were present in the free-base form. Rearing pots were cleaned daily, and fresh food was added. The percentage of surviving larvae, larval development times, and weights of pupae (sexed after the emergence of adults) were recorded. The percentage of

surviving larvae was correlated with scopolamine concentrations by Sperman rank correlation and also compared by comparison for multiple proportions (Zar, 1999). The other two variables, larval development times and weight of pupae, were tested by one-way and two-way ANOVA, respectively, with a *post hoc* Tukey test (Zar, 1999).

Allocation of scopolamine in B. suaveolens One month after the transference to pots, four clones of each of the 10 genotypes, in cutting stage, were planted randomly in the experimental field of the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, at a spacing of 2×2 m, and they received normal agricultural treatments (irrigation, weeding, and fertilization). In each of the phenological stages (cuttings, 1 month after the transference to the field; vegetative stage, 3 to 6 months; flowering stage, and fruiting stage, more than 6 months) one clone of each genotype was harvested and its organs [roots, fully expanded young leaves (from first to fourth leaves), old leaves (from eighth leaf), stems, flowers, and unripe fruits, when present] were separated and dried at 40±2°C in a mechanical convection oven, ground in a mill, and stored at −5°C for extraction of scopolamine. At the stage of flowers, four genotypes were lost in the extraction procedure. The concentration of scopolamine was compared within phenological stages by one-way ANOVA with a *post hoc* Tukey test (Zar, 1999).

Costs of induction of scopolamine To assess the effect of an increase of scopolamine content in leaves of *B. suaveolens* on fitness, we conducted an experiment where we increased the scopolamine production by the wound hormone methyl jasmonate. We compared leaf-relative growth rate (LRGR) of control and treated plants. Nine 3-month-old genotypes of *B. suaveolens* (three clones for each individual) were subjected to 3 different treatments (modified from Baldwin, 1998) in a controlled environment (see conditions above): (a) 5.0 μg of methyl jasmonate (MeJA—Aldrich™) suspended in 10 ml of water was applied with a syringe inserted below the soil surface at the base of the shoot, with 5 ml applied to each side of each plant; (b) 50 μg MeJA as above; and (c) control with 10 ml distilled water was applied as above. Twelve days after application, leaves were sampled, and the concentration of scopolamine was measured as below. LRGR [LRGR=[((final leaf area 12 d after the application − initial leaf area)/initial leaf area)×100] for each treatment was calculated by measuring the initial and final leaf areas, with a Licor LI-3000 leaf area meter (Li-Cor Biosciences). Both scopolamine concentration and LRGR were compared between treatments by one-way ANOVA, with a *post hoc* Tukey test (Zar, 1999).

Induced response of scopolamine in leaves by leaf artificial damage Fourteen days after the development of the first 16 leaves, six genotypes growing in 2-l pots (five clones per genotype), maintained in an acclimatized chamber as above, were submitted to artificial damage as follows: At time zero, five clones of each genotype had half their leaf area removed with a pair of scissors (hereafter, distal half leaf or undamaged leaf) and were harvested and stored until chemical analysis (see below). At time zero, and after 24, 48, 96, and 192 hr (concerning to first to fifth clones, respectively), the remaining half leaf (hereafter, proximal half leaf or damaged leaf) was harvested. Both proximal and distal half leaves were dried individually in an oven at 50°C for 2 d, ground to a powder, and maintained at −10°C until extraction and measurement of scopolamine (see below). The induction ratio, which expresses how scopolamine varies after the artificial damage, was calculated by dividing the concentration of scopolamine at any time treatment (0, 24, 48, 96, or 192 hr) in the proximal half leaf (artificial damage treatment) by the concentration of scopolamine at time zero in distal half leaf (control). The half leaf was delimited by half of

the length along the principal vein. The areas of distal leaf halves (76.70±5.91 cm, $\overline{X} \pm SE$), measured with a Licor LI-3000 leaf area meter (Li-Cor Biosciences), were not significantly different from the area of proximal leaf halves (77.81±6.47 cm) (paired *t*-test, $t=0.905$, $df=9$, $P=0.389$). Because scopolamine concentration in *B. suaveolens* did not show significant hourly fluctuations (one-way ANOVA, $F_{11,72}=0.324$, $P=0.977$), the experimental design as used by van Dam et al. (1993) was not necessary. Preliminary experiments did not show significant differences in scopolamine content between proximal (1.76±0.15 mg/g) and distal (1.71±0.16 mg/g) leaf halves (paired *t*-test, $t=0.3620$, $df=9$, $P=0.726$) and, therefore, no correction in the calculation of the induction ratio was necessary. To compare the induction ratio at different times, one-way repeated-measure ANOVA, with a *post hoc* Tukey test, was used (Zar, 1999).

Induced resistance to a generalist herbivore To determine whether artificial damage altered resistance (*sensu* Karban and Baldwin, 1997) of plant leaves against generalist herbivores, we use as model the fourth instar of the polyphagous noctuid moth *S. frugiperda* (see above). Larvae were reared as above and allowed to starve for 24 hr. After starvation, larvae received food *ad libitum* in three different treatments during 24 hr in an acclimatized chamber (L12:D12, 25±1°C, and about 70% RH) as follows: (1) control: intact leaves of *Ricinus communis* (Euphorbiaceae) that did not show negative effects on the larval performance of *S. frugiperda* in previous work (Ferraz, 1991), (2) distal halves of leaves of *B. suaveolens* (see above), and (3) proximal half leaves of *B. suaveolens*, 24 hr before harvesting (see above). Only one genotype of *B. suaveolens* was used to carry on this assay. This individual had 0.14 mg/g of scopolamine in the distal half and 0.37 mg/g of scopolamine in proximal half leaves. To assess the role of undamaged (distal half) and damaged (proximal half) leaves of *B. suaveolens* on larval performance of *S. frugiperda* in relation to control leaves of *R. communis*, the relative consumption rate (RCR) and relative growth rate (RGR) were calculated following Miller and Feeny (1983) and Blau et al. (1978). Compounds in *B. suaveolens* in damaged or undamaged leaves may alter the consumption rate and the growth rate of *S. frugiperda*. When growth rate is plotted against consumption rate, a positive slope is expected if no inhibitory or toxic compounds are present. If compounds are toxic, a higher RCR would result in an even lower growth rate, and a negative slope is expected. If compounds inhibit feeding, RCR is expected not to be related to RGR (Blau et al., 1978). To compare the regression lines, we first tested the significance of each one; because the regression line of treatment 2 was not significant (see Fig. 2), ANCOVA was not done (Zar, 1999). Instead, one-way ANOVA, with a *post hoc* Tukey test, was carried out by comparing either RCR or RGR among the treatments (Zar, 1999).

Extraction, GC-flame ionization detector and GC-MS analysis of scopolamine Samples of plant material (~2.0g) were homogeneized in 5 ml EtOH and centrifuged at 4,000 rpm for 10 min. This procedure was repeated ×3, and the EtOH supernatants were combined and evaporated under vacuum. TAs were purified from the EtOH extract by acid–base work up and analyzed by GC-MS as given in Freitas et al. (1996). For scopolamine quantification, a GC-flame ionization detector (FID) analyses was carried out by using a Hewlett Packard 5890 GC with a FID coupled with a Hewlett Packard (HP 3396-A) electronic integrator, with SA-1 (100% polydimethyl siloxane) capillary column (30 m×0.25 mm×0.25 μm). The GC-FID conditions were as follows: injection temperature 250°C; temperature program 150–300°C, 4°C/min; split ratio 1:100; and carrier gas He 1 ml/min. An aliquot of 1 μl of the alkaloidal extracts was redissolved in 200 μl of a 2-mg/ml MeOH solution of stearic

acid methyl ester was injected as an internal standard. We used methyl stearate as the internal standard because its retention time (RT) did not overlap the RT of the TAs, used as a commercial alkaloid (the pyrrolizidine monocrotaline).

Results and Discussion

Performance of larvae of S. frugiperda on scopolamine diet Diets containing scopolamine affected the larvae of *S. frugiperda* negatively. Developmental time increased with greater scopolamine concentration in the diet (Table 1), and the percentage of individuals surviving on diets was negatively affected by an increase in scopolamine concentrations (Table 1). Concentrations of 0.25% dry weight were effective, and these are comparable with scopolamine concentrations found in plant parts (Fig. 1). Weight of pupae for both sexes decreased marginally with an increase of scopolamine concentration; there was no interaction between sex and diets (Table 1).

Although the generalist noctuid *S. frugiperda* does not share its evolutionary history with *B. suaveolens*, based on the results with this generalist herbivore model, we can suggest that scopolamine may be a factor acting as antifeeding on generalist insects. TAs have been reported to be effective against many generalist herbivorous insects (Hsiao and Fraenkel, 1968; Krug and Proksch, 1993; Wink, 1993; Shonle, 1999), but none of these shared their evolutionary history with the bioassayed host plants. Additionally, other TAs are presented in *B. suaveolens*, but in lower concentration than scopolamine (Freitas et al., 1996), and the role of these in plant resistance remains to be studied. Does this compound also act against generalist insects that share an evolutionary history with *B. suaveolens* or as specialists? To our knowledge, only two specialist butterflies feed on leaves of *B. suaveolens*, *P. euryanassa* and *M. cymothoe*; the former sequesters TAs from leaves, but the latter does not (Freitas et al., 1996), and no record of generalists in the field has been observed by us. Specialists can be attracted to a compound that formally may have been a feeding deterrent for generalists (van der Meijden, 1996, and see references in Shonle and Bergelson, 2000), and although our data suggest an antiherbivore defense against specialists by scopolamine, it remains an open question whether this compound or other TAs in general act as an antiherbivore defense against specialists.

Allocation of scopolamine in B. suaveolens The scopolamine concentration changed according to the organs investigated and with the phenological stage of the plant (Fig. 1). In the cutting stage, young leaves and stems had a higher concentration than roots (one-way ANOVA, $F_{2,27}=59.497$, $P<0.001$). In the expanded leaf stage, stems had a higher concentration than other plant parts ($F_{3,36}=117.828$, $P<0.001$). In the flowering stage, the highest scopolamine concentrations were found in the flowers, young leaves, and stems; old leaves and roots had the lowest ($F_{4,25}=188.340$, $P<0.001$). In the fruiting stage, scopolamine had the highest concentrations in unripe fruits ($F_{5,54}=400.679$, $P<0.001$).

The allocation of scopolamine to different tissues of *B. suaveolens* in different phenological states is as predicted by optimal-defense theory: more valuable structures, in terms of fitness, receive greater defenses (McKey, 1974, 1979; Rhoades, 1979). Young leaves, flowers, and mainly unripe fruits with seeds have progressively higher scopolamine concentrations than other tissues. Zangerl and Bazzaz (1992) suggest that fruits; seeds; and, to a lesser extent, flowers, are more heavily defended than vegetative plant parts. This differential allocation of potential defensive compounds has been found in many plants. Van Dam et al. (1996) found that young leaves of *Cynoglossum officinale* contain higher

Table 1 Performance of *Spodoptera frugiperda* fed on artificial diets with different concentrations of scopolamine

	Control (0%)	0.05%	0.25%	0.5%	2.5%	Statistical Tests
Survivorship (%)	96 a	96 a	84 b	54 c	32 d	Sperman correlation $rs=-0.975$, $t=-7.550$, $P=0.005$. Comparison for multiple proportions $\chi^2=40.873$, $df=4$, $P<0.001$
Developmental time (days)[a]	15.20±0.12 a (24)	19.13±0.18 b (24)	20.62±0.22 c (21)	24.00±0.32 d (13)	24.00±0.00 d (08)	One-way ANOVA $F_{4,85}=299.863$, $P<0.001$ (*post hoc* Tukey test)
Male pupae weight (mg)	0.184±0.002	0.175±0.004	0.183±0.008	0.172±0.008	0.161±0.006	Two-way ANOVA
Female pupae weight (mg)	0.193±0.006	0.190±0.005	0.207±0.006	0.178±0.006	0.185±0.009	Between scopolamine concentrations $F_{4,68}=2.188$, $P=0.080$. Between sexes $F_{1,68}=9.606$, $P=0.003$. Interaction $F_{468}=9.606$, $P=0.624$

For each variable, different letters mean significant differences at 5%.

[a] The number in parentheses represents the sampled number.

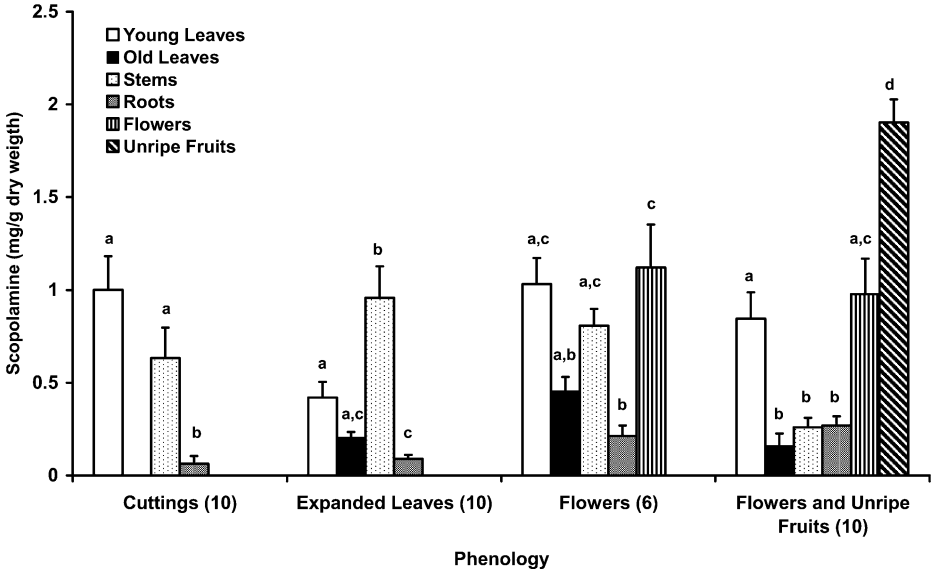


Fig. 1 Concentration of scopolamine (mean ± SE, mg/g dry weight) of different organs in different phenological stages of *Brugmansia suaveolens*. One-way ANOVA was performed within each phenological state. Different letters mean significant differences at 5% (*post hoc* Tukey test)

concentrations of pyrrolizidine alkaloids than old leaves. Wink (1985) reported that fruits in *Lupinus polyphyllus* had about 100 times more quinolizidine alkloids than other tissues. The same pattern was also found for furanocoumarins in *Pastinaca sativa*; the concentration of xanthotoxin being highest in fruits, lowest in roots, and intermediate in leaves (Zangerl and Rutledge, 1996, and references therein). Shonle (1999) found that scopolamine reduced the growth of the generalist *Spodoptera exigua*, but did not have any effect on either the generalist *Heliothis zea* or the specialist *Manduca sexta*. No natural generalist or specialist herbivore insect sharing an evolutionary history with *B. suaveolens* was tested in our work, but based on literature (see above) and these results (see Table 1), the suggestion is that this compound (maybe together with the other TAs) protects *B. suaveolens* against generalist natural enemies. However, what about ripe fruits? A high content of distasteful compounds in ripe fruits would be deterrent for dispersers. Cipollini and Levey (1997) and Cipollini (2000) suggested that when seeds from ripe fleshy fruits are dispersed by vertebrates, the allocation of distasteful compounds in their pulp represents a compromise between defensive and attractive functions (defense-tradeoff hypothesis). Nevertheless, *B. suaveolens*, which is common on the edge of rivers, has a ripe dry dehiscent fruit, and the seeds are water-dispersed (J.R. Trigo, personal observation). In this case, distasteful compounds, if present in ripe fruits, do not represent any ecological costs for this species because they do not affect disperser vertebrates.

Costs of induction of scopolamine At 50 μg/plant, the MeJA application significantly increased the concentration of scopolamine in leaves of *B. suaveolens* to about 9.5 times the concentration in controls (one-way ANOVA, $F_{2,24}$=15.670, P<0.001, Fig. 2). Similarly, these plants showed a leaf production decrease by a factor of 2.3 times, and this is significantly lower than that of control plants (one-way ANOVA, $F_{2,24}$=9.142, P=0.001,

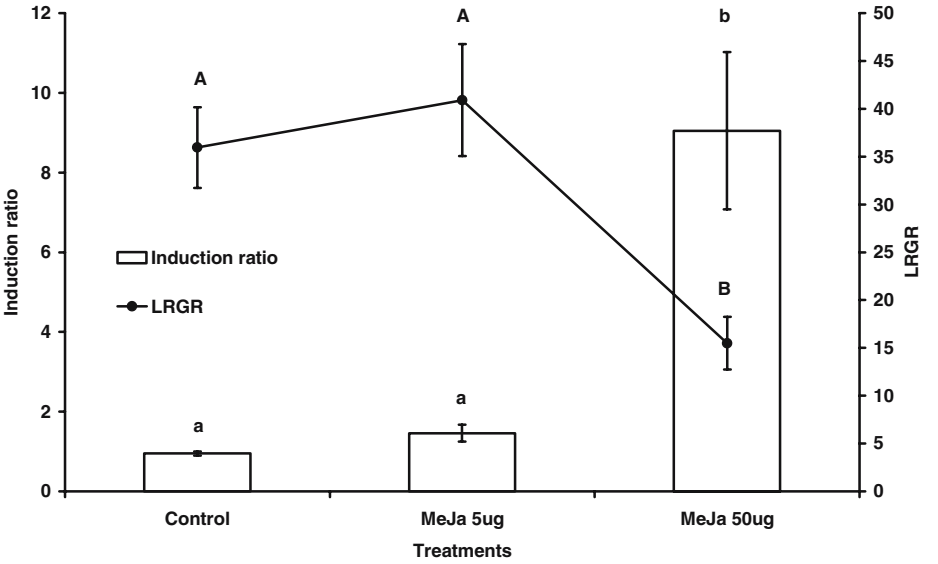


Fig. 2 Induction ratio in leaves and LRGR ($\overline{X} \pm SE$) of *Brugmansia suaveolens* with roots treated with MeJA. For each variable, different letters mean significant differences at 5% (*post hoc* Tukey test)

Fig. 2). A lower concentration of MeJA (5 μg/plant) neither increased the concentration of scopolamine nor showed significant differences in LRGR in relation to control plants (Fig. 2).

The treatment with MeJA hormone increased the production of scopolamine and decreased the LRGR in *B. suaveolens*. MeJA is known to inhibit protein biosynthesis, which is essential for growth, and acts as elicitor for several secondary metabolic pathways

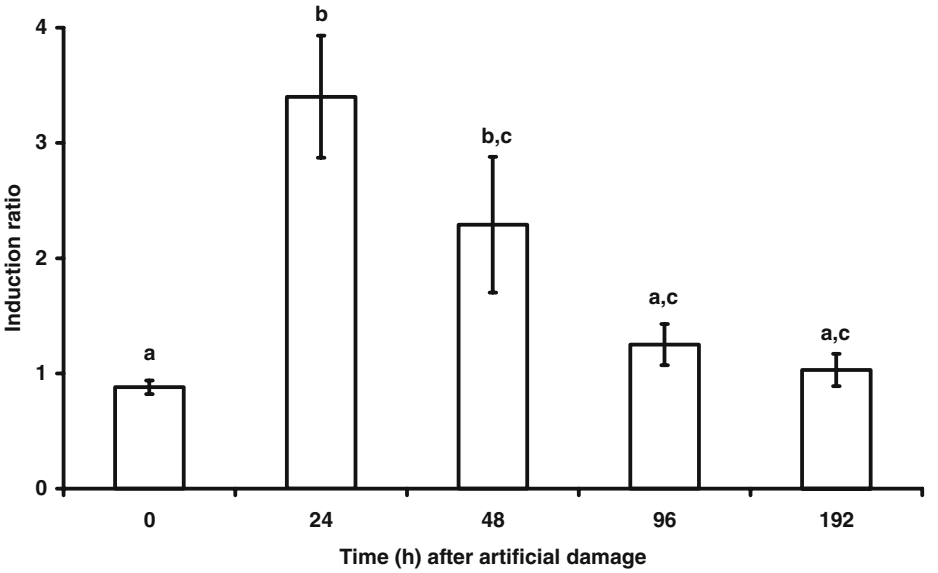


Fig. 3 Induction ratio ($\overline{X} \pm SE$) in leaves of *Brugmansia suaveolens* at different times after artificial damage. Different letters mean significant differences at 5% (*post hoc* Tukey test)

Table 2 RCR and RGR ($\overline{X} \pm SE$) of *Spodoptera frugiperda* larvae in a 24-hr feeding trial on distal (undamaged) and proximal (artificially damaged) halves of leaves of *Brugmansia suaveolens*

Treatments	RCR	RGR
Control (leaves of *Ricinus communis*) (n=33)	0.424±0.109[a]	0.193±0.048[a]
Undamaged leaves (n=24)	1.379±0.106[b]	−0.102±0.022[b]
Artificial damaged leaves (n=24)	1.380±0.092[b]	−0.246±0.022[c]
One-way ANOVA	$F_{2,78}$=29.801, P<0.001	$F_{2,78}$=38.520, P<0.001

For each variable, different letters mean significant differences at 5% (*post hoc* Tukey test).

(see Reinbothe et al., 1994 for a review). As discussed by Baldwin (1998), this experiment showed a decrease in growth due to jasmonate induction, but it is not clear which jasmonate-inducible responses were responsible for the growth reduction. Scopolamine induction was a component of this response. Although LRGR may only be an indirect measure of fitness, with seed production and/or seed viability being a better measure, a reduced foliage production in the field might seriously alter competition for light and therefore have an impact on fitness. Induced plant responses to herbivory are considered to be cost-saving strategies, as pointed out by optimal-defense theory, but costs are hard to detect because multitrophic interactions involving many trade-offs may participate in the plant environment (Dicke and van Loon, 2000; Heil and Baldwin, 2002; van Dam et al., 2003).

Induced response of scopolamine in leaves by leaf artificial damage The highest induction (3.40±0.54 times) was recorded 24 hr after artificial damage; subsequent periods showed a rapid decrease in induction rate, reaching the control level at 96 hr (one-way repeated-

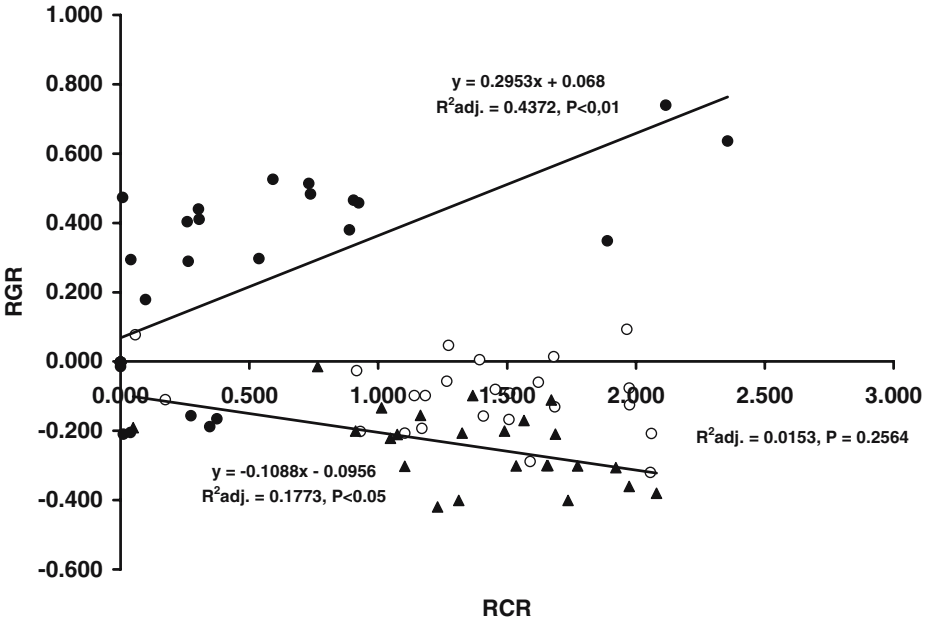


Fig. 4 RGR as a function of RCR for the fourth larval instar of *Spodoptera frugiperda* fed with *Ricinus communis* undamaged leaves (control, *black circles*) and undamaged (*white circles*) and artificially damaged (*triangles*) leaves of *Brugmansia suaveolens*. The regression line was shown only when it was significant

measure ANOVA, $F_{4,20}$=10,103, P<0.001, Fig. 3). Leaves of *B. suaveolens* increase their content of scopolamine after artificial damage. This induced response has been demonstrated for different organs in several species (see Karban and Baldwin, 1997, for a review). The highest induction rate for *B. suaveolens* was found 24 hr after the damage; after this, the rate decreased to constitutive levels. This relaxation timing has also been described for several species, and the duration of the phenomenon varies within hours or days after damage (Green and Ryan, 1972; Underwood, 1998) or even years (Haukioja, 1980). Nevertheless, Shonle and Bergelson (2000) reported no induction of TAs after damage in the taxonomically close *D. stramonium*. They analyzed damaged leaves 5 d after the damaging treatments; in this period, the level of alkaloids may have already decreased to their constitutive levels. Rapid induction after damage followed by a rapid relaxation to constitutive levels follows the predictions of optimal-defense theory, and may save costs associated with self-toxicity and biosynthesis of high levels of defensive substances (Karban and Baldwin, 1997).

Induced resistance to larvae of S. frugiperda due to mechanical damage of B. suaveolens Feeding on intact leaves of *B. suaveolens* had an adverse effect on larvae of the generalist noctuid moth *S. frugiperda*. Although the RCR increased in relation to control diet (1.379±0.106), the RGR showed significant negative values (−0.102±0.022). RGR became even more negative when *S. frugiperda* fed on artificially damaged leaves (−0.246±0.022), although it remained similar to that on intact leaves (1.380±0.092) (Table 2, Fig. 4).

The diet of undamaged leaves of *B. suaveolens* showed a negative effect on the RGR of the generalist insect *S. frugiperda*. Artificially damaged leaves increased this effect. There is a reduction in RGR, but not in RCR, when the diets with undamaged and artificially damaged leaves are compared (Table 2). As pointed out by Blau et al. (1978), this must occur when ingested compounds are toxic to herbivores. Compounds present in undamaged *B. suaveolens* leaves may be toxic for herbivorous insects and the toxic effect may have increased after artificial damage. Nevertheless, we cannot exclude that the toxic effect may also be due to compounds other than scopolamine or TAs. Artificial damage can also lead to the induction of compounds such as phenolics, terpenes, and defense-related proteins (Karban and Baldwin, 1997). Additionally, the impact of induced response on natural populations of herbivores, either specialists or generalists, such as population fluctuations and regulation (Haukioja, 1980; Edelstein-Keshet and Rausher, 1989; Lundberg et al., 1994), or the increase of induced susceptibility in herbivores counteracting the benefits of induced response (Underwood, 1998), remains to be investigated.

Acknowledgments We thank Flávia Tavares Colpas, Keith S. Brown, Klaas Vrieling, Rodrigo Cogni, Suzana de Fátima Alcântara, and two anonymous reviewers for comments on the manuscript. The research was supported by a Fundação de Amparo à Pesquisa do Estado de São Paulo grant for JRT (98/01065-7). We thank also the Prefeitura Municipal de Jundiaí for permission to work in Serra do Japi. This work is part of the Ph.D. dissertation of MNA at Pós-Graduação em Biologia Vegetal, Instituto de Biologia, Universidade de Campinas.

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J Chem Ecol (2007) 33:311–317
DOI 10.1007/s10886-006-9228-3

Tolerance of *Drosophila* Flies to Ibotenic Acid Poisons in Mushrooms

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Received: 23 May 2006 / Accepted: 20 November 2006 /
Published online: 29 December 2006
© Springer Science + Business Media, LLC 2006

Abstract The mushroom genus *Amanita* has a spectrum of chemical compounds affecting survival and performance of animals. Ibotenic acid is one of such compounds found in some *Amanita* mushrooms. We studied the effects of ibotenic acid and its derivative, muscimol, on egg-to-pupa survival, pupation time, and pupal size in five *Drosophila* species (Diptera: Drosophilidae), *Drosophila bizonata*, *Drosophila angularis*, *Drosophila brachynephros*, *Drosophila immigrans*, and *Drosophila melanogaster*. The first three species are mycophagous and use a wide range of mushrooms for breeding, whereas *D. immigrans* and *D. melanogaster* are frugivorous. We reared fly larvae on artificial medium with 500, 250, 125, and 62.5 μg/ml of ibotenic acid and/or musimol. The three mycophagous species were not susceptible to ibotenic acid, whereas the two frugivorous species were affected. In experiments with *D. melanogaster*, muscimol was less toxic than ibotenic acid.

Keywords Ibotenic acid · *Amanita* · *Drosophila* · Susceptibility · Mycophagy

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Introduction

A wide variety of secondary metabolites occur in fungi (Martin, 1979), many of which are potentially harmful to insects that consume them. Several are toxic to both invertebrates and vertebrates. Besl et al. (1987) studied the effects of 127 species of fungi on the development of *Drosophila melanogaster* Meigen and observed that six of eight *Amanita* (Agaricales, Amanitaceae) species impaired the development of the fly. In contrast, mycophagous species, *Drosophila kuntzei* and *Drosophila phalerata*, were not susceptible to amatoxins of *Amanita phalloides* (Fr.) Link (Besl et al., 1987). Jaenike et al. (1983) studied mycophagous and nonmycophagous species of *Drosophila* for tolerance to α-amanitin, a potential inhibitor of RNA polymerase II, and observed that mycophagous species were tolerant, whereas nonmycophagous species were intolerant.

We studied the effects of ibotenic acid and its derivative, musimol, found in *Amanita pantherina* (DC.:Fr.) Krombh.; *Amanita ibotengutake* T. Oda, C. Tanaka & Tsuda; and *Amanita muscaria* (L.: Fr.) Pers. on survival and performance of mycophagous (*Drosophila bizonata* Kikkawa and Peng, *Drosophila angularis* Okada, and *Drosophila brachynephros* Okada) and nonmycophagous species (*Drosophila immigrans* Sturtevant and *D. melanogaster*). Among them, *D. bizonata* was observed to breed abundantly on *A. pantherina* and *A. ibotengutake* in Kyoto, central Japan (Yamashita et al., unpublished data). The three mycophagous species use a wide variety of mushroom species for breeding, whereas *D. immigrans* and *D. melanogaster* are frugivorous, although *D. immigrans* sometimes feeds and breeds on mushrooms (Kimura et al., 1977; Nishiharu, 1980; Tuno, 2001; Takahashi et al., 2005).

Materials and Methods

Flies Laboratory stocks of *D. bizonata*, *D. angularis*, *D. immigrans*, and *D. melanogaster* were established from females collected in Tokyo, central Japan, and a stock of *D. brachynephros* was from females collected in Sapporo, northern Japan. They were maintained on poison-free laboratory medium for at least three generations before the test.

Experiments Test medium was prepared as follows. A mixture of cornmeal (2.5 g), malt (3.5 g), sugar (5 g), dry yeast (3 g), and agar (1 g) was added to water to make up 60 ml. Thirty grams of ground commercial mushroom [*Flammulia velutipes* (Curt.: Fr.) Sing.] were added. The medium was boiled for 15 min and 0.45 ml of propionic acid was added. To this medium, ibotenic acid (Tocris Cookson, St. Louis, MO, USA) or muscimol (0.25 H2O; Wako Chemical, Osaka, Japan) dissolved in NaOH (0.1 N) solution was added to make final concentrations of 500, 250, 125, and 62.5 μg/ml. The concentration of NaOH in the test medium was standardized to 0.01 N by the addition of NaOH solution. The rearing of larvae was made by using vials (6 ml vol) with test medium (440 or 1,000 μl) and a cotton plug.

Eggs were collected from laboratory fly stocks, sterilized in 70% ethanol for 15 min, and placed in vials with test medium (ten eggs per vial). The vials were kept at 23°C. Five to ten replicates were used. When pupae were formed in the vials, they were counted daily to determine pupation time (i.e., days from egg to pupation), and then they were measured for size.

Two experiments were conducted. In experiment 1, the effects of ibotenic acid and muscimol were compared with *D. melanogaster*; in experiment 2, the effect of ibotenic acid on four different *Drosophila* species was investigated. The effect of the chemicals on the

egg-to-pupa survival was analyzed using logistic regression; the effect on pupation time was analyzed using Cox's regression analysis (Cox, 1972); the effect on pupal size was determined with standard regression analysis. If a significant effect was observed in these regression analyses, pairwise comparison was made between experimental treatments and control; i.e., values at 62.5, 125, 250, or 500 μg/ml value in control. The pairwise comparison was made using Fisher's exact test for survival, with Cox's regression analysis for pupation time, and Dunnett's test for pupal size. The sequential Bonferroni correction was made for the results of these multiple comparisons. Statistical analyses were performed using JMP version 5.0.1.2 (SAS Institute, Cary, NC, USA).

Results

Table 1 shows the effects of ibotenic acid and muscimol on egg-to-pupa survival, pupation time, and pupal size in *D. melanogaster*. The effects of these two chemicals on egg-to-pupa survival and pupation time were significant (logistic regression for survival, $P<0.001$; Cox's regression for pupation time, $P<0.001$); i.e., survival decreased and pupation time was prolonged with the increase of concentration. According to pairwise comparisons with control, the effect of ibotenic acid became significant at a concentration of 62.5 μg/ml,

Table 1 Effects of ibotenic acid and muschimol on egg-to-pupa survival (a), pupation time (b) and pupal size (c) in *Drosophila melanogaster*

Conc. (μg/ml)	Ibotenic acid				Muscimol			
	Mean		SD	*N*	Mean		SD	*N*
Number of pupae emerged per 10 eggs planted[a]								
500	0.1**	±	0.3	10	1.5**	±	1.4	10
250	0.2**	±	0.4	10	4.3**	±	1.5	10
125	0.3**	±	0.5	10	7.3**	±	0.9	10
62.5	6**	±	2.5	5				
25–50					8.4	±	0.7	9
0 (control)	8.3	±	0.7	16				
Pupation time (days)[b]								
500	15.0**			1	14.7**	±	2.7	15
250	11.0**	±	0.0	2	7.9**	±	0.5	43
125	6.7**	±	0.6	3	6.7	±	1.7	70
62.5	6.9*	±	1.3	60				
25–50					6.1	±	0.3	186
0 (control)	6.4	±	0.5	136				
Pupal size (mm)[b]								
500	2.8			1	2.9	±	0.1	13
250	2.9	±	0.0	2	3.0	±	0.1	43
125	2.9	±	0.1	3	2.9	±	0.2	67
62.5	2.9	±	0.1	60				
25–50					3.0	±	0.1	88
0 (control)	3.0	±	0.1	129				

*$P<0.05$ significantly different from control, **$P<0.001$ significantly different from control

[a] N = number of replicates, each of which comprised 10 eggs

[b] N = Number of fly individuals

Table 2 Effects of ibotenic acid on egg-to-pupa survival, pupation time, and pupal size in mycophagous and frugivorous species

Species	Feeding habit	Ibotenic acid concentration (μg/ml)													
		0 (control)			62.5			125			250			500		
		Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Number of pupae emerged per 10 eggs planted[a]																
D. bizonata	M	4.3	1.1	7										3.9	0.7	7
D. angularis	M	5.6	1.7	10	6.0	2.9	10	4.4	1.9	10	6.2	2.8	10	4.6	2.4	10
D. brachynephros	M	6.6	1.3	10	7.7	2.2	10	2.9	1.6	10	4.8	1.8	10	6.5	1.7	10
D. immigrans	F	7.0	1.1	10	6.3	2.7	10	4.7*	1.6	10	2.5**	1.5	10	0.0**	0.0	10
D. melanogaster	F	8.3	0.7	16	6.0**	2.5	5	0.3**	0.5	10	0.2**	0.4	10	0.1**	0.3	10
Pupation time (days)[b]																
D. bizonata	M	13.2	1.7	31										12.3	1.5	26
D. angularis	M	12.9	1.1	56	13.4	1.8	58	12.0	1.8	40	15.7	0.9	62	13.6	1.0	46
D. brachynephros	M	11.9	0.8	66	14.0	1.0	72	12.3	1.3	30	13.5	0.5	54	13.3	1.1	52
D. immigrans	F	11.2	0.3	70	11.6ns	0.5	63	13.0*	0.8	47	18.7**	1.7	25			
D. melanogaster	F	6.4	0.5	136	6.9*	1.3	60	6.7**	0.6	3	11.0**	0.0	2	15.0**		1
Pupal size (mm)[b]																
D. bizonata	M	2.4	0.1	29										2.4	0.1	26
D. angularis	M	2.4	0.1	54	2.4	0.1	54	2.5	0.1	39	2.4	0.1	43	2.5	0.1	46
D. brachynephros	M	2.4	0.1	65	2.4	0.1	71	2.5	0.1	28	2.5	0.1	47	2.4	0.1	51
D. immigrans	F	3.6	0.2	69	3.7	0.2	60	3.7	0.1	46	3.8	0.1	14			
D. melanogaster	F	3.0	0.1	129	2.9	0.1	60	2.9	0.1	3	2.9	0.0	2	2.8		1

M = mycophagous, F = frugivorous

*$P<0.05$ significantly different from control, **$P<0.001$ significantly different from control

[a] *N* = Number of replicates, each of which comprised ten eggs

[b] *N* = Number of fly individuals

Table 3 Results of analysis on the effect of ibotenic acid on egg-to-pupa survival, pupation time, pupal size in mycophagous (M) and frugivorous (F) species

	Species				
	D. bizonata	*D. angularis*	*D. brachynephros*	*D. immigrans*	*D. melanogaster*
Feeding habit	M	M	M	F	F
Egg-to-pupa survival					
Logistic regression analysis					
R square	0.003	0.002	0.007	0.089	0.490
Chi square	0.471	1.448	4.650	87.760	91.640
P	NS	NS	NS	<0.001	<0.001
Pupation time					
Cox's regression analysis					
Chi square	0.195	0.647	2.546	49.873	45.630
P	NS	NS	NS	<0.001	<0.001
Pupal size					
Regression analysis					
t ratio	−0.300	1.920	0.570	2.170	−2.480
P	NS	NS	NS	NS	0.043

whereas the effect of muscimol became significant at a concentration of 125 μg/ml in survival and at a concentration of 250 μg/ml in pupation time (two-tailed Fisher's exact test on survival, $P<0.05$; Cox regression analysis on pupation time, $P<0.01$) (Table 1). Survival was significantly lower in individuals treated with ibotenic acid than in those treated with the same concentration of muscimol at a concentration of 125 μg/ml or higher (two-tailed Fisher's exact test, $P<0.001$). Ibotenic acid seemed to cause high mortality in the earlier developmental phases, because almost no trace of crawling or feeding of larvae was observed at concentrations of 125, 250, and 500 μg/ml. On the other hand, the effects of ibotenic acid and muscimol on pupation time were different only at a concentration of 250 μg/ml or higher (Cox's regression analysis, $P<0.001$). On pupal size, ibotenic acid had only a weak effect (regression analysis, $P=0.043$) and muscimol had no significant effect (regression analysis, $P>0.05$).

Table 2 shows the effects of ibotenic acid on four other *Drosophila* species, *D. bizonata*, *D. angularis*, *D. brachynephros*, and *D. immigrans* (the results on *D. melanogaster* are also shown as a reference), and Table 3 shows the results of regression analyses. Ibotenic acid did not show any detrimental effect on mycophagous species *D. bizonata*, *D. angularis*, and *D. brachynephros*. In contrast, *D. immigrans* was affected by this chemical, although less seriously than *D. melanogaster*; the effects on egg-to-pupa survival and pupation time on *D. immigrans* were significant at a concentration of 125 μg/ml or higher (Fisher's exact test on survival, $P<0.05$, Cox's regression on pupation time, $P<0.05$; Table 2). In this species, the effect of ibotenic acid on pupal size was not significant (Dunnett's test, $P>0.05$).

Discussion

Tsunoda et al. (1993) reported that fruit bodies of *A. muscaria*, a species closely related to *A. pantherina* and *A. ibotengutake*, on average, contain ibotenic acid at a concentration of 343 μg/ml. In this study, it appeared that ibotenic acid had no effects on survival, pupation

time, or pupal size in mycophagous *Drosophila* species, *D. bizonata*, *D. anguralis*, and *D. brachynephros*, even at a concentration of 500 μg/ml. In contrast, it had deleterious effects on frugivorous species, *D. melanogaster* and *D. immigrans*, at concentrations of 125 or 250 μg/ml. Thus, ibotenic acid in mushrooms may have a defensive function at least against nonmycophagous insects. On the other hand, muscimol was less harmful than ibotenic acid. Muscimol, detected at low concentrations, is assumed to be an inevitable byproduct of ibotenic acid. Tsunoda et al. (1993) reported that fruit bodies of *A. pantherina* and *A. ibotengutake* contain this chemical at a concentration of 22 μg/ml, but Stømer and Janak (2004) failed to detect muscimol from fresh fruit bodies of *A. muscaria*. Thus, it is not likely that muscimol has a defensive function.

It is unclear why mycophagous *Drosophila* species have evolved such high tolerance to ibotenic acid. These species use a wide variety of mushrooms (Kimura et al., 1977; Nishiharu, 1980; Tuno, 2001; Takahashi et al., 2005), and mushrooms containing ibotenic acid (e.g., *A. pantherina* and *A. ibotengutake*) are expected to be only a small part of their food resources. Therefore, they would be able to maintain their populations without *Amanita* mushrooms.

A similar situation has been reported in American mycophagous drosophilids. They are highly tolerant to α-amanitin, another poison of *Amanita* mushrooms that is harmful to all kinds of eukaryotes, although they use a wide variety of mushrooms lacking α-amanitin (Jaenike, 1985). Jaenike (1985) argued that a primary selective advantage of α-amanitin tolerance is that it allows drosophilids to breed in mushrooms that are free of nematodes. Nematodes are abundantly found in many fungi, and they infest drosophilids, breeding on them and rendering them infertile. However, nematodes are absent in mushrooms containing amanitin.

Amanita mushrooms may avoid predation because they may appear to generate ibotenic acid. However, mycophagous maggots are often seen feeding on mushroom tissue without disturbing the growth of mushrooms. It is possible that maggot attack has little effect on host fitness. Poisonous mushrooms may serve as a refuge for mycophagous flies that are less harmful to host fungi. We need to investigate animal consumption of mushrooms in nature to clarify the relevance of mushroom poisons.

Acknowledgement We thank Dr. John Romeo for his patient help and advice. We thank Prof. M. T. Kimura for improving the manuscript and the members of the Department of Ecology and Genetics, Graduate School of Environmental Earth Science, Hokkaido University, for their technical support. This work is supported in part by Grants-in-Aid (15380104 and 17405030) for Scientific Research (B) to C. Tanaka from the Japan Society for the Promotion of Science and the COEs for Microbial-Process Development Pioneering Future Production Systems and Innovative Food and Environmental Studies Pioneered by Entomomimetic Sciences (the COE programs of the Ministry of Education, Culture, Sports, Science and Technology, Japan).

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J Chem Ecol (2007) 33:319–329
DOI 10.1007/s10886-006-9234-5

Spectrum of Cyanide Toxicity and Allocation in *Heliconius erato* and *Passiflora* Host Plants

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Received: 2 April 2006 / Accepted: 27 November 2006 /
Published online: 3 January 2007
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Abstract The larvae of three races of *Heliconius erato* were fed various species of *Passiflora* containing varying levels of cyanoglucosides. The mortality rate of larvae and pupae rose when larvae were fed species of *Passiflora* capable of releasing larger quantities of cyanide. When larvae were fed species of *Passiflora* with these properties, the resulting adult butterflies also released higher levels of cyanide. This may serve as a defense mechanism. The compounds responsible for the release of cyanide were not evenly distributed throughout the adult butterfly's body. The thorax contained the highest concentration of cyanogenic substances, followed by the head, wings, and abdomen. The younger tissues of *Passiflora* plants had higher levels of cyanide-releasing compounds than stems and mature leaves. Cyanogenic glycoside distribution within the plants is consistent with optimal allocation theory. The levels of cyanide-releasing substances in plants varied depending on the season.

Keywords *Heliconius erato cyrbia* · *Heliconius erato favorinus* · *Heliconius erato demophoon* · *Passiflora* · Cyanogenic glycosides · β-glucosidase · Cyanide · Ecuador · Peru · Panama

Introduction

The genus *Heliconius* comprises a large group of tropical butterflies whose larvae specialize in feeding upon members of the *Passiflora*. Within this genus, *Heliconius erato* is a common species that inhabits second growth and disturbed forest of Central and South

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America and is adapted to altitudes ranging from sea level to 1,600 m. *Heliconius erato* has radiated into numerous geographic races that are members of local mimicry groups. Each race feeds upon a different species of *Passiflora*, and the plants are, to a greater or lesser extent, cyanogenic. The adult butterflies are unpalatable to predators (Chai 1996) due to the accumulation of cyanogenic glycosides as larvae (Spencer 1988). Cyanogenesis has been found in both insects and plants containing cyanogenic glycosides that break down upon tissue injury to yield cyanide. Cyanide is released when the enzyme β-glucosidase hydrolyzes a cyanogenic glycoside-generating cyanohydrin, which then rapidly breaks down at normal cell pH to a carbonyl compound and cyanide. In addition, some tissues contain the enzyme α-hydroxynitrile lyase that acts upon the cyanohydrin to release cyanide and the aglycone (Höesel 1981).

In *Heliconius* butterflies, cyanogenic compounds are found in the cuticular part of the integument (Nahrstedt and Davis, 1983, 1985; Davis and Nahrstedt, 1985). The location of β-glucosidase is unknown, but it is separated from the cyanoglucoside (Nahrstedt and Davis, 1983). All stages in the life history of Heliconiinae have been examined, and the highest concentrations of cyanide-releasing compounds occur in the egg stage. The concentrations in the larval and pupal stages do not vary greatly from each other, but cyanogenic compound concentrations increase in adults as a result of cyanoglucoside biosynthesis during the adult stage (Nahrstedt and Davis, 1985).

Potential variations in levels of cyanogenic glycoside concentrations within body parts of *Heliconius* adults, as related to their host plants, has not been previously explored. For this paper, levels of cyanide liberated from different body parts of various races of *H. erato* that were fed natural *Passiflora* host plants were examined. Analyses of cyanogenesis were performed over two seasons (summer and fall) on separate parts of *Passiflora* plants (young leaves, mature leaves, and stems). Roots, fruits, and flowers were not included in the analysis because *Heliconius* larvae rarely feed upon these tissues. Cyanide liberated from plant tissues was correlated with the cyanide liberated from tissues of adults that fed on the plants. The discussion addresses the costs and benefits of allocating cyanogens as a defense mechanism to different body parts of this species and the implications of these variations on the palatability of this butterfly and on its mimetic relationships.

Methods and Materials

Plant Growth and Insect Rearing This study was conducted at the University of Florida in Gainesville with butterfly colonies that were raised in Lord & Burnham glass houses (5×8 m). *Heliconius erato favorinus* was collected in Tingo Maria, Huanuco, Peru, located on the eastern side of the Andes, whereas *Heliconius erato cyrbia* was collected in Pichincha, Ecuador, located on the western side of the Andes, and *Heliconius erato demophoon* in Soberania National Park, Panama.

Most of the *Passiflora* plants began as cuttings that were propagated in pots for use in this research. All vouchers for *Passiflora* plants used for this study are deposited at the Missouri Botanical Garden, St. Louis, MO, and identification of the plants was confirmed by Dr. John MacDougal, an expert in systematics of the family Passifloraceae.

Larvae, Experimental Design, and Treatments *Heliconius erato favorinus*, *H. e. cyrbia*, and *H. e. demophoon* larvae were raised in environmental chambers under controlled temperature (27°C), humidity (78%), and photoperiod (14 hr light/10 hr dark). The three races were fed only their natural host plants. All of these host plants possess complex

cyclopentenoid glycosides (Olafsdottir et al., 1989; Hay-Roe 2004). *Heliconius erato favorinus* (*N*=19, 13 males and 6 females) was fed *Passiflora trifasciata*. *Heliconius erato cyrbia* (*N*=14, seven males and seven females) was fed *Passiflora punctata* and *Passiflora rubra* (*N*=12, six males and six females), and *H. e. demophoon* (*N*=10, five males and five females) was fed *Passiflora biflora*. Males and females of *H. e. cyrbia* and *H. e. favorinus* were examined separately, and for each specimen, the head, thorax, abdomen, and wings were examined separately. The head, thorax, and abdomen of *H. e. demophoon* were examined as a unit but the wings separately. Excreta from caterpillars that were fed different host plants were also examined for cyanogenesis.

Temporal Cyanide Quantification of Passiflora Plants *Passiflora* plants were examined during the summer (July–August) and fall (September–October) of 1999 (Table 1). All plants were potential host plants of *H. erato* in different geographical areas of Brazil, Costa Rica, and Ecuador. The analyses were performed on young leaves, mature leaves, and stems.

All analyzed *Passiflora* plants were subjected to the same environmental conditions (humidity, temperature, and natural photoperiod) in the greenhouses. None of the plants examined was exposed to herbivores.

Quantitative Determination of Cyanide Cyanogenesis was quantified by the Lambert procedure (Lambert et al., 1975) as modified by Brinker and Seigler (1989, 1992). In short, plant or butterfly samples were ground to a fine powder in liquid nitrogen. The powdered plant or butterfly tissue was added to a 10-ml vial containing 0.1 M phosphate buffer (pH 6.8). A 5-ml vial containing fresh 1 M NaOH solution was then placed inside the 10-ml vial, and the 10-ml vial was stoppered tightly. Samples were prepared quickly to avoid loss of HCN. Vials were incubated at 37°C overnight. During incubation, HCN released from tissue was trapped in the NaOH solution, and formed NaCN.

After incubation, cyanide was quantified in a spectrophotometer (Spectro 22; Labomed, Culver City, CA, USA) after a sequential addition of a series of reagents detailed in Brinker and Seigler (1989). Addition of exogenous β-glucosidase [linamarase from flax seed, courtesy of David Jones; sulfatase type IV from limpets (Sigma, St. Louis, MO, USA); almond-β-glucosidase (Sigma); and β-glucosidase that we extracted from *P. biflora* and *Passiflora coriacea*] was used when samples initially tested acyanogenic. A standard curve was prepared following the methods of Brinker and Seigler (1989).

Table 1 *Passiflora* plants examined for cyanogenesis in this study

Subgenus	Subgenus Section	*Passiflora* Species	Country of Origin	*N*	
				Summer	Fall
Decaloba (Plectostemma)	Decaloba	*biflora*	Costa Rica	22	12
	Decaloba	*misera*	Brazil	12	6
	Decaloba	*punctata*	Ecuador	6	0
	Decaloba	*trifasciata*	Peru	17	6
	Auriculata	*auriculata*	Ecuador	22	10
	Xerogona	*capsularis*	Brazil	6	14
	Xerogona	*rubra*	Ecuador	12	0

All plants are used by *H. erato* in different geographical areas

After cyanide release and assay, the remaining plant or insect tissue was dried at 65°C to constant weight (approx. 6 d) and weighed. Dry weight was used to calculate the micrograms of CN^- released per gram of dry weight of plant tissue or butterfly body tissue.

Statistical Analysis Two-way analysis of variance was performed with the statistical program SYSTAT 10. Some of the data were not normally distributed, and the data were subjected to square root transformation to compensate for increasing variation associated with larger mean values (Zar 1996). However, the statistical results with transformed data did not vary significantly from the raw data, so the results of the raw data are reported here. A Tukey test was used for multiple comparisons. Linear regression was used to test the correlation of cyanide released by butterfly tissues with cyanide released by tissues of *Passiflora* host plants.

Results

Cyanide Quantification in H. erato Fed Natural Host Plants The amount of cyanide released per given weight of tissue varied among the races (F=29.90; df=1, 139; P<0.001). *Heliconius erato cyrbia* released greater amounts of cyanide than *H. e. favorinus*. There were differences in cyanide released between the sexes (F=8.26; df=1, 139; P<0.005) and by different body parts (F=8.14; df=3, 139; P<0.001).

Differences in host plant utilization resulted in differences in cyanide released from individuals of *H. e. cyrbia* fed on *P. rubra* and *P. punctata* (F=10.02; df=1, 97 P<0.002). When *H. e. cyrbia* fed on *P. rubra* (Fig. 1), the butterflies released greater amounts of cyanide per gram of tissue compared to butterflies fed on *P. punctata* (Figs. 1 and 2). Males and females of this race differed in the quantity of cyanide released (F=5.68; df=1, 97; P< 0.025). Females concentrated higher amounts of cyanide-releasing compounds in their bodies when fed *P. rubra* as larvae. Furthermore, the cyanogenic compounds were stored differently within the body parts (F=7.32; df=3, 97; P<0.001) (Fig. 1), with higher levels

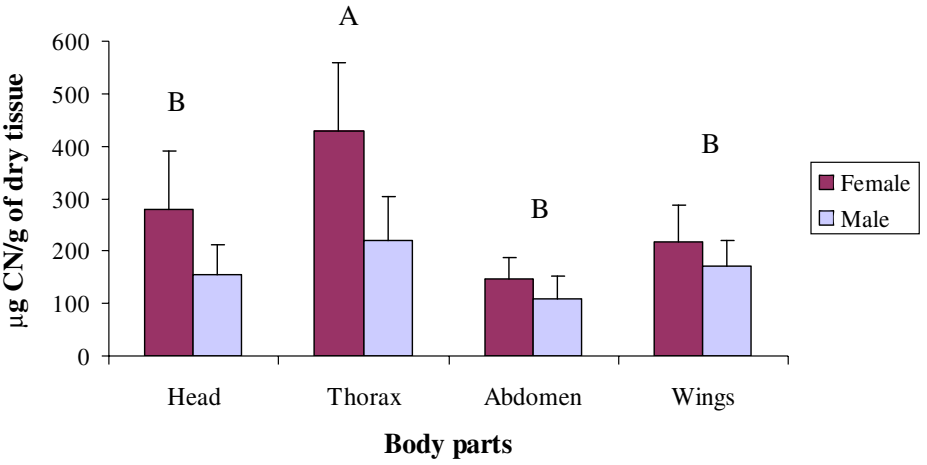


Fig. 1 Cyanide liberated from body parts of male and female *H. e. cyrbia* fed *P. rubra*. *Different letters* indicate significant differences between means (Tukey HSD-test). *Bars* indicate standard error

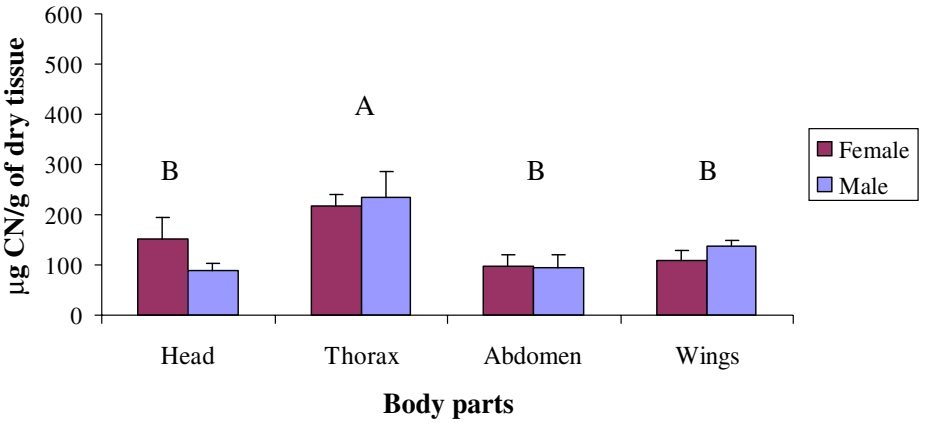


Fig. 2 Cyanide liberated from body parts of male and female *H. e. cyrbia* fed *P. punctata*. *Different letters* indicate significant differences between means (Tukey HSD-test). *Bars* indicate standard error

of cyanide released from the thorax. When *H. e. cyrbia* was fed *P. punctata* (Fig. 2), cyanide concentrations released from the sexes did not differ. The thorax and the head released greater amounts of cyanide per unit weight of tissue than the abdomen and wings (F=12.31; df=3, 52; P<0.001) (Fig. 2).

Low levels of cyanide were released from the body of *H. e. favorinus* fed *P. trifasciata*, and there were no differences in cyanide-releasing activity in the male and female thorax and head. The wings and abdomen released the lowest concentrations. More males than females were examined because more than 50% of the males examined were negative. Only five males and four females were cyanogenic, and the addition of exogenous β-glucosidase did not promote the release of cyanide, indicating that cyanide release was not due to the

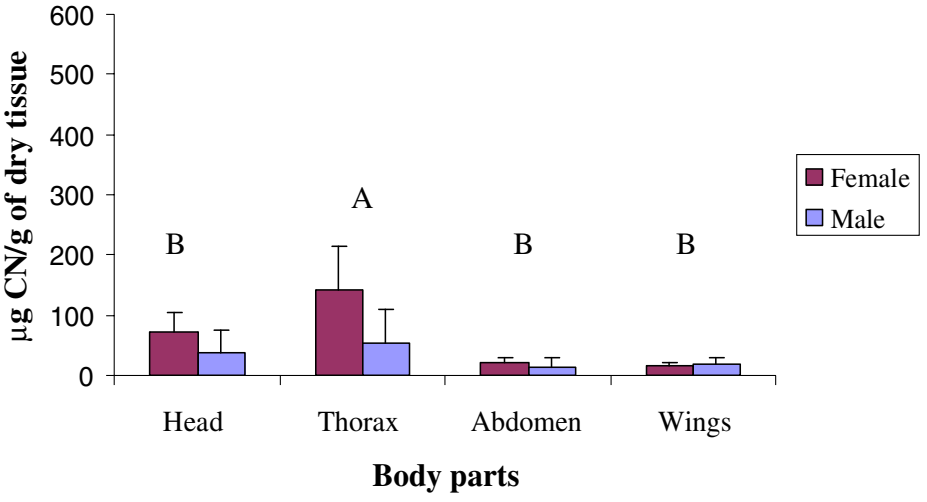


Fig. 3 Cyanide liberated from body parts of male and female *H. e. favorinus* fed *P. trifasciata*. *Different letters* indicate significant differences between means (Tukey HSD-test). *Bars* indicate standard error

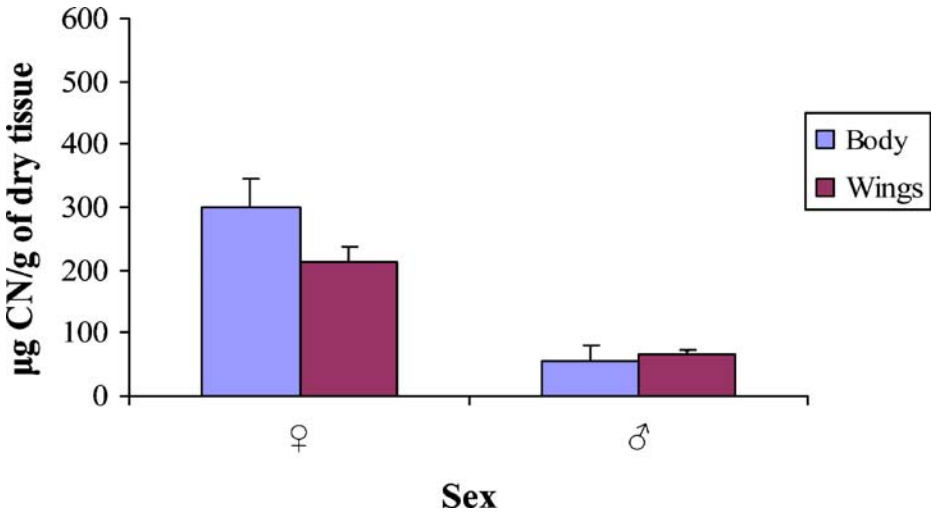


Fig. 4 Cyanide liberated from male and female *H. e. demophoon* fed *P. biflora*. *Bars* indicate standard error

lack of the enzyme. The most probable reason for lack of cyanide release was a very low, or no, amount of cyanogenic glycosides within the butterfly's body (Fig. 3).

Low levels of cyanide release were found in male *H. e. demophoon* fed on *P. biflora*, its natural host plant. Furthermore, cyanide concentrations varied between body parts. In general, the body (which in these samples includes the head, thorax, and abdomen) released more cyanide per unit of tissue than the wings (F=7.21; df=9,19; P=0.003) (Fig. 4).

Mortality of H. erato Fed Natural Host Plants High mortality during the larval stage was observed in *H. e. cyrbia* fed *P. rubra*. In all cases, larvae died during the early instars (Table 2). During the pupal stage, 12% of the individuals fed as larvae on *P. punctata* died. For the rest of the treatments, mortality rate was low. *Heliconius erato favorinus* showed no mortality when fed *P. trifasciata*.

Temporal Variation of Cyanide Release in Passiflora Plants The amount of cyanide released per weight of plant tissue varied between the two analyzed seasons (F=129.95; df=1, 388; P<0.001) (Fig. 5a and b). In the fall, cyanide released per unit tissue increased to approximately double the amount released in summer. *Passiflora rubra* was not examined in the fall; however, *Passiflora capsularis*, a close relative (Table 1), follows this

Table 2 Mortality in different stages of the life history of *H. e. favorinus*, *H. e. cyrbia*, and *H. e. demophoon* feeding on natural host plants

Race of *H. erato*	Total *N*	Host plant	Larvae		Pupae		Adult	
			N dead	% dead	*N* dead	% dead	*N* dead	% dead
favorinus	25	*P. trifasciata*	0	0	0	0	0	0
cyrbia	25	*P. punctata*	1	4.0	3	12.0	0	0
	48	*P. rubra*	20	41.7	2	4.2	2	4.2
demophoon	25	*P. biflora*	1	4.0	0	0	0	0

same pattern of variation in toxicity and possesses the same cyanogenic glycoside as *P. rubra* (Hay-Roe 2004). There was a difference in cyanide-releasing capacity between host plants examined (F=45.06; df=6, 388; P<0.001) (Table 1) and plant parts (F=131.83; df= 2, 388; P<0.001). The release of cyanide was always higher from new leaves, and this value differed significantly from the cyanide released from mature leaves and stems. The cyanide-releasing capacity of mature leaves and stems did not follow a constant pattern and varied according to the species (Fig. 5a, b).

The regression line in Fig. 6 indicates that 68% of the variation in cyanide concentration released from tissue of *Heliconius* butterflies is explained by the ability of their host plants to release cyanide, which we assume to be due to cyanogen concentration in the host plant tissues.

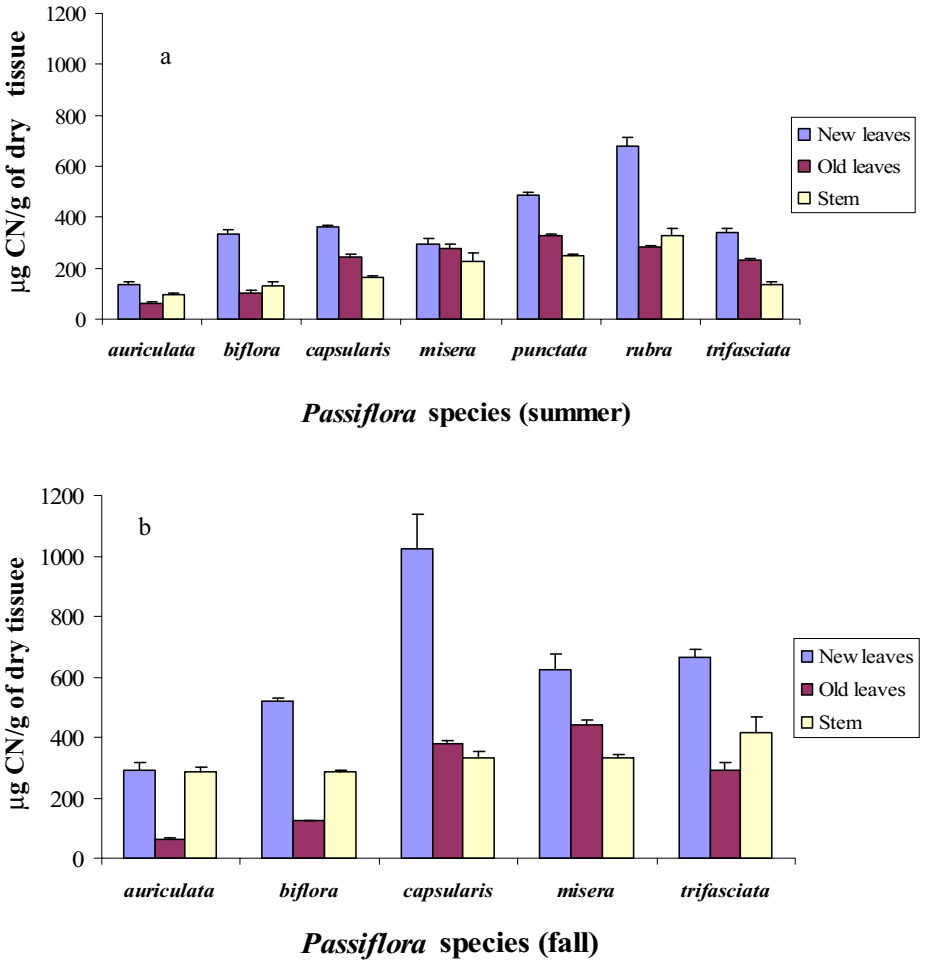


Fig. 5 Cyanide liberated from *Passiflora* plants in the **a** summer and **b** fall seasons. *Bars* indicate standard error

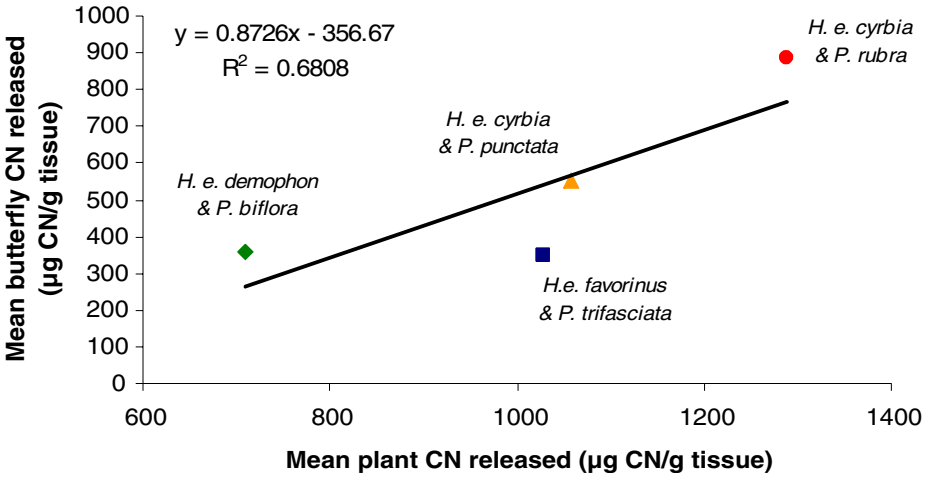


Fig. 6 Regression of the mean cyanide released from *H. erato* vs. the mean cyanide released from the natural *Passiflora* host plants upon which larvae fed

Discussion

Cyanogenic Concentrations in H. erato Fed Passiflora Host Plants and its Implications for Palatability The host plant consumed was the main reason for the variation in cyanide released by different races of *H. erato*. Different levels of cyanogenic compounds in the larvae might be due to both differing levels of cyanogenic substances in the host plants eaten and to detoxification mechanisms in the larvae (Hay-Roe 2004). The thorax and the head were the body parts that released greater amounts of cyanide per unit weight of tissue, which suggests greater accumulation of cyanogenic compounds in these body parts. These results did not follow the patterns of cardenolide accumulation reported for monarch butterflies, which possess higher concentrations of cardenolides in wings (Brower and Glazier, 1975). Low levels of cyanide released from the wings and abdomen might be the result of low levels of hemolymph-containing β-glucosidase in the former and low levels of sclerotized cuticle in the latter.

Cyanogenesis in *Heliconius* butterflies probably functions as an alert signal to predators to reinforce the memory of toxicity, also called aide-mémoire mimicry (Rothschild 1984). Under this assumption, it is not necessary for selection to favor highly cyanogenic wings because low levels of HCN will be enough to trigger the recall of experiences of the bad taste and smell of HCN and aglycone compounds formed during cyanogenesis. A naive predator can quickly learn to avoid feeding on that particular pattern of wing coloration (Chai 1988) and flight (Srygley and Chai, 1990), and the members that constitute a Müllerian ring will benefit from it. The selection for belonging to the ring's common pattern will be reinforced (a similar mechanism of avoidance is likely found for the larval stage; however, a definitive test was not run). Achieving a high degree of unpalatability has a negative aspect, as was demonstrated by the mortality rates. Members of *H. e. cyrbia* fed with *P. rubra* benefit by becoming distasteful, but there was a high cost paid for this benefit: individuals feeding on *P. rubra* had a higher mortality rate and spent a longer period feeding on the host (Hay-Roe 2004), which increases the time they are exposed to

parasitoids. At least for *H. e. cyrbia*, there is a limiting range of potential toxicity to a predator because of high mortality at the larval stage caused by ingested cyanide. On average, adult butterflies that fed as larvae on cyanogenic plants are slightly smaller in size than comparable butterflies feeding on other less-cyanogenic host plants (Hay-Roe 2004).

Heliconius erato favorinus seems to be better adapted to detoxifying and tolerating different chemicals in *Passiflora* plants (Hay-Roe 2004), probably due to more efficient degradative enzymatic activity, or by possessing higher tolerance of chemicals, for example, by having a target-site insensitivity (Brattsten, 1979; Lindroth and Weisbrod, 1991). Differences between strategies for coping with cyanogenic compounds probably reflect genetic variation among the races of *H. erato*. Some insects, for example, *Trichoplusia ni* and *Heliothis virescens*, excrete ingested nicotine rapidly as a detoxification mechanism. We did not find cyanide released from excreta of the caterpillars examined in this study.

Temporal Variation in Cyanide in Passiflora Plants Considerable variation in cyanide released was found among *Passiflora* examined over two seasons (summer and fall). The amount of cyanide released per gram in the fall was almost double the amount observed in the summer season. Variation in cyanogenic glycosides has been linked to herbivore presence (Cooper-Driver et al., 1977) and to altered climatic conditions such as temperature (Ellis et al., 1977; Rammani and Jones, 1985; Jones and Rammani, 1985). In this study, the biotic and abiotic conditions were similar for all analyzed *Passiflora* species in the greenhouse because temperature and light were the same for all plants. An environmental factor that might be responsible for the variation is the difference in photoperiod due to seasonal change. A study of seasonal variation in flavonoid concentration performed on temperate-zone *Alliaria* plants (Haribal and Renwick, 2001) showed that flavonoid concentrations were lower in the summer and higher in the fall. It would be interesting to see how this variation in cyanogenic glycoside concentration correlates to the survival rates of larvae and/or adults of *Heliconius* species.

Higher release of cyanide occurred from the new growth of *Passiflora* plants and followed the same pattern of variation between the summer and fall seasons. Similar temporal variation of cyanogen accumulation in passionvines could occur during the dry and rainy seasons in the tropical rainforest. The distribution of cyanogens within the plant is consistent with optimal allocation theory (McKey 1974), which states that vulnerable plant tissues and organs are defended more than older senescing ones. It has been demonstrated elsewhere that for several cyanogenic species, young developing tissues contain the highest amounts of cyanogenic glycosides (Conn 1981).

Implications of Cyanogenic Compounds for Mimicry Different precursors of toxic substances within a butterfly's body produce a palatability spectrum (Brower et al., 1967), which in turn affects the conditioning of avian predators towards their prey (Brower et al., 1967; Pough et al., 1973). *Heliconius erato* and its comimic *Heliconius melpomene* are members of a Müllerian mimicry ring of races in the South and Central America rainforests. There is some disagreement as to whether *erato* or *melpomene* is the model for this ring, but Mallet (1999) listed three different reasons for favoring *erato* as the model. The first is that *erato* is more abundant in the areas where it coexists with *melpomene*; second, *erato* has a broader geographical distribution; and third, *erato* has a broader habitat than *melpomene*.

If *H. e. favorinus* in Tingo Maria, Huanuco, is less palatable, then this species is, in fact, a weak model. We should then expect that this would directly affect the presence of the less

common *H. melpomene*. This would be true if *P. trifasciata* were the only host plant used by *H. e. favorinus* in the area. It was certainly the most common in Tingo Maria. However, *Passiflora auriculata* (which is the host plant of *Heliconius sara*), *Passiflora laurifolia*, *Passiflora tricuspis*, *P. rubra*, and *Passiflora vespertilio* have been found in Huanuco (Brako and Zarucchi, 1993) and are potential host plants for *H. e. favorinus*.

Recently, doubts have been raised with regard to the existence of the classical Müllerian mimic. Instead, some have proposed a new term, "quasi-Batesian mimicry" (Huheey 1976; Speed and Turner, 1999), as an alternative explanation for some types of polymorphism associated with mimicry. Quasi-Batesian mimicry states that if members of a Müllerian mimicry complex are not equally distasteful, then the presence of individuals that are less distasteful would lead to an increase in predation on the models, and this will result in an overall weakening of the Müllerian mimicry. In this model, predator learning and memorization of the degree of distastefulness of the prey is assumed. However, more recent models have argued that the assumptions that lead to quasi-Batesian mimicry may not be met in most real situations, casting doubt on the model (Mallet and Joron, 1999; Kokko et al., 2003).

Males of *H. e. favorinus* that fed on *P. trifasciata* had a very low ability to release cyanide. They probably get protection from the presence of more unpalatable conspecifics (females or other conspecifics that do not feed on *P. trifasciata*). It is likely that females will be more affected by potential male palatability because females are probably more exposed to predators, particularly during oviposition and while searching for host plants.

Acknowledgements This study constitutes part of a University of Florida doctoral dissertation. MMHR thanks the following for help and encouragement: T. C. Emmel, F. Slansky, H. McAuslane, and D. Jones as members of my committee; C. Jiggins and M. Beltran for hospitality while in Panama; the Delores A. Auzenne Graduate Scholars Fellowship, the James & Margaret Gahan Scholarship, and the John A. Mülrennan Senior Scholarship at the University of Florida for financial support; Butterfly World; and the Association of Tropical Lepidoptera for travel grants to Tingo Maria, Huanuco, Peru, and Panama. We thank the Instituto de Recursos Naturales, Ministerio de Agricultura, Peru; the Smithsonian Tropical Research Institute, Panama; and the United States Department of Agriculture for collecting, exporting, and importing permits.

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J Chem Ecol (2007) 33:331–343
DOI 10.1007/s10886-006-9216-7

Jasmonate, Salicylate, and Benzoate in Insect Eggs

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Received: 4 April 2006 / Revised: 18 August 2006 / Accepted: 10 October 2006 /
Published online: 27 December 2006
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Abstract Jasmonic acid (JA) and salicylic acid (SA) are key molecules in the initiation of plant defensive responses to attack by herbivores and pathogens, respectively. Our previous work has shown that JA occurs at high concentrations in eggs and neonates of lepidopteran species. Here, we extend our analyses to eggs of 15 non-lepidopteran insect species spanning eight orders, again screening for JA, but also including SA and one of its metabolic precursors, benzoic acid. We detected all three compounds in eggs of almost all the species examined. Moreover, concentrations of these compounds were variable across species, suggesting that species accumulate and/or utilize the compounds differently. Eggs of the fruit-feeding fly *Rhagoletis pomonella* contained the greatest concentrations of all three compounds, which appear to be common in fruit. The presence of these plant-derived compounds in eggs may serve defensive or other functions for insects, and could conceivably trigger plant defensive responses after oviposition.

Keywords Benzoic acid · Jasmonic acid · Salicylic acid · Phytohormone · Plant defenses

Introduction

The phytohormones jasmonic acid (JA) and salicylic acid (SA) initiate plant defensive responses to attack by herbivores and pathogens (Walling, 2000). Jasmonate is derived from linolenic acid via the octadecanoid pathway and activates defensive genes that initiate "induced systemic resistance" against some insect herbivores and the release of volatile compounds that attract natural enemies to herbivore-infested plants (Walling, 2000). Salicylate appears to be produced by at least two pathways, one via phenylalanine, cinnamic acid, and benzoic acid (BA), and the other via isochorismate (Métraux, 2002; Delaney, 2005). Salicylic acid initiates defensive response against pathogens and some insect herbivores resulting in "systemic acquired resistance" (Walling, 2000; Métraux, 2002). Molecules of both JA and SA can be produced at sites of insect damage and are

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ingested by feeding herbivores, activating detoxification genes that appear to provide "early-warning" signals indicating imminent production by plants of toxic defense compounds (Li et al., 2002).

We recently reported that lepidopteran eggs and neonates can harbor high concentrations of JA (Tooker and De Moraes, 2005). We detected JA in all nine lepidopteran species that we screened. This JA may have been derived from the larval diets of ovipositing mothers, although the presence in two species of significantly greater quantities of JA in unfed neonates than in eggs suggests that some lepidopteran species may synthesize JA *de novo*. Because of the consistent presence of JA in lepidopteran eggs and neonates, we postulated that JA may play an unknown functional role in lepidopterans and, perhaps, in insects in general (Tooker and De Moraes, 2005).

In this paper, we expand on our previous analyses of JA in insect eggs, extending our analyses to non-lepidopteran eggs and screening for SA and one of its metabolic precursors, BA. As with JA, the presence of these plant-derived compounds in insect eggs seems counterintuitive because of their roles in initiating plant defensive responses (Walling, 2000; Delaney, 2005). Previous studies have shown that eggs laid on plants can trigger a hypersensitive response (Shapiro and DeVay, 1987; Balbyshev and Lorenzen, 1997; Garza et al., 2001), which is SA-mediated (McDowell and Dangl, 2000; Walling, 2000; Ollerstam and Larsson, 2003); however, to our knowledge, there have been no previous reports of SA in insect eggs. Similarly, we are unaware of any investigations of BA in insect eggs despite its being a component of defensive secretions in some insect species and of sex pheromones in others (e.g., Classen and Dettner, 1983; Nishida et al., 1996). We present data from analyses of eggs from 15 insect species in eight orders. We report levels of JA, BA, and SA for eggs of individual species and also analyze data based on groupings of species with similar trophic positions, diets, or feeding styles.

Methods and Materials

Insect and Diet Samples We obtained eggs of 15 species representing 8 insect orders (Table 1). Two species were field collected, and the exact composition of their diets is unknown. The other 13 species were colony reared on plants or artificial diets, and we were able to obtain samples of diet for 10 of these 13 (Table 1). We collected 50–250 mg of each diet (five samples per diet) or eggs directly into FastPrep® tubes (Qbiogene, Carlsbad, CA, USA) containing 1 g of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA) and froze them at −80 °C until processing (see below). Because of the low mass of eggs (<1 mg for most species), we combined multiple eggs (2–20 depending on mass) into single samples, processing 5–10 samples for each species (total eggs analyzed per species: 10–100). For the phasmid *Extatosoma tiaratum* (MacLeay), eggs were large enough (~40 mg each) to process only two eggs per sample (we analyzed 10 eggs), and for the cockroach *Periplaneta americana* L., we processed one ootheca per sample, and each ootheca contained 16 eggs (we analyzed 80 eggs). For the cecidomyiid *Mayetiola destructor* (Say), eggs were so small that we were unable to obtain accurate masses even when 20 eggs were combined in each sample; therefore, we were unable to include this species in some of our statistical analyses (see below).

Extraction and Quantification of JA, BA, and SA To extract and detect JA, BA, and SA, we used a previously described method (Tooker and De Moraes, 2005) modified slightly from

Table 1 Insect species whose eggs were analyzed for jasmonate, benzoate, and salicylate

Insect Order	Insect Family and Species	Common Name	Source	Diet	Larval Habit
Phasmida	Phasmatidae; *Extatosoma tiaratum* (MacLeay)	MacLeay's spectre	Penn State Univ.	*Pyracantha* sp.[a]	Chewing[b]
Orthoptera	Mantidae; *Tenodera aridifolia* Saussure	Chinese mantid	Centre Co., PA[c]	Insect prey	Predator
Blattaria	Blattidae; *Periplaneta americana* L.	American cockroach	Carolina Bio. Supply	Artificial diet[a]	Omnivore
Isoptera	Termopsidae; *Zootermopsis nevadensis nevadensis* (Hagen)	Dark pacific coast dampwood termite	Univ. Maryland	Rotting wood	Deadwood feeder
Homoptera	Pentatomidae; *Nezara viridula* L.	Southern green stink bug	Univ. Palermo (Italy)	Seasonal vegetables[a]	Sucking[b]
	Lygaeidae; *Oncopeltus fasciatus* Dallas	Large milkweed bug	Carolina Bio. Supply	Sunflower seeds[a]	Sucking[b]
Neuroptera	Chrysopidae; *Chrysoperla carnea* (Stephens)	Green lacewing	Gardens Alive	Artificial diet	Predator
Diptera	Cecidomyiidae; *Mayetiola destructor* (Say)	Hessian fly	Penn State Univ.	*Triticum aestivum* L.	Sucking[b]
	Tephritidae; *Rhagoletis pomonella* (Walsh)	Apple maggot	NY State Ag. Res. Sta.	Apples[a]	Fruit feeder
	Anastrepha ludens (Loew)	Mexican fruit fly	USDA, Edinburg, TX	Artificial diet[a]	Fruit feeder
Coleoptera	Chrysomelidae; *Diabrotica virg. Virgifera* LeConte	Western corn rootworm	USDA, Brookings, SD	Artificial diet[a]	Chewing[b]
	D. virg. Zeae Krysan & Smith	Mexican corn rootworm	USDA, Brookings, SD	Artificial diet[a]	Chewing[b]
	D. barberi Smith & Lawrence	Northern corn rootworm	USDA, Brookings, SD	Artificial diet[a]	Chewing[b]
	D. balteata LeConte	Banded cucumber beetle	USDA, Brookings, SD	Artificial diet[a]	Chewing[b]
Hymenoptera	Apidae; *Apis mellifera* L.	Honey bee	Centre Co., PA[b]	Floral resources	Nectar/ pollen

[a] Diet analyzed for JA, BA, and SA (see Table 2).

[b] Herbivore with chewing or sucking mode of feeding.

[c] Field collected.

Schmelz et al. (2003, 2004). Briefly, we derivatized carboxylic acids to methyl esters, which were isolated by using vapor phase extraction and analyzed by GC-MS with isobutane chemical ionization with selected-ion monitoring. We quantified amounts of methyl jasmonate (meJA), methyl salicylate (meSA), and methyl benzoate (meBA) by using standard curves made with the pure compounds (Sigma-Aldrich, St. Louis, MO, USA) and relying on internal standards to confirm derivatization and recovery. We also processed samples without the derivatization agent to verify that the meJA, meBA, and meSA we recovered were not themselves present in eggs, but were derived from the carboxylic acids. To confirm the identity of meJA, meBA, and meSA in our samples, we analyzed extracts by GC-MS with electron ionization with selected-ion monitoring of characteristic fragments by comparing retention times and spectra with those of pure compounds (*m*/*z* ratio of characteristic fragments—meJA: 27, 41, 67, 83, 151, 224; meBA: 51, 77, 105, 136; meSA: 15, 39, 65, 92, 120, 152). We used electron ionization in the "selected-ion" mode rather than the "scan" mode because mass spectrometry using the former mode has greater sensitivity.

Statistical Analyses We analyzed amounts of JA, BA, and SA on a "per-gram-fresh-weight" basis as well as a "per-egg" basis because of disparate egg masses ranging from <0.01 mg (*M. destructor*) to ~40 mg (*E. tiaratum*). To stabilize variance, we added one to each value to control for zeros and square-root transformed the data, making between-species comparisons of JA, BA, and SA levels and egg mass by analysis of variance (ANOVA) and comparing individual means with Tukey's honestly significantly different (HSD) means separation test (Sokal and Rohlf, 1995; Statistix, 2003). Because JA, BA, and SA in insect eggs are likely to be derived from the nymphal or larval diet of the ovipositing mothers (Tooker and De Moraes, 2005), we also used ANOVA to compare levels of JA, BA, and SA (transformed as above) in diets and among 4 feeding-based groupings of the species (sucking herbivores, chewing herbivores, fruit feeders, and predators; Table 1). Not all species were represented in this latter analysis because we only used functional groupings comprising at least two species. Lastly, we used regression analysis to test for linear relationships between variables (Sokal and Rohlf, 1995; Statistix, 2003).

Results

We detected JA, BA, and SA in eggs of almost all the insect species and diet samples that we screened, with the exceptions that eggs of the seed bug *Oncopeltus fasciatus* Dallas did not yield SA, and its diet yielded neither SA nor JA. Retention times and selected-ion mass spectra of meJA, meBA, and meSA recovered from eggs and diets were nearly identical to those of pure standards, confirming the identity of JA, BA, and SA in our samples. Moreover, we did not recover the 3 compounds in absence of the derivatization agent, verifying that the methyl esters we measured were derivatized from JA, BA, and SA and were not themselves present in samples.

For the diets we were able to analyze, we found significant differences across the diets for all three compounds (Table 2). The diet of *E. tiaratum*, *Pyracantha coccinea* M. Roemer (Rosaceae), contained the greatest amount of JA, which was significantly greater than all of the other diets (Tukey's HSD $P<0.05$). The remaining diets were statistically similar and intermediate in concentration relative to *P. coccinea* and the sunflower-seed diet

Table 2 Concentrations of JA, BA, and SA in insect diets

Insect Species	Diet	ng JA/g Diet[a]	ng BA/g Diet[a]	ng SA/g Diet[a]
E. tiaratum	*Pyracantha coccinea* M. Roemer	213.7±58.0 a	6606±1630 b	161.9±89.7 b
P. americana	Artificial diet[b]	42.7±11.1 b	9608±1628 b	3653±420 a
N. viridula	Fava beans	15.9±5.4 bc	8154±2403 b	10.5±7.2 b
O. fasciatus	Raw sunflower seeds	0±0 c	9598±1669 b	0±0 b
R. pomonella	Red Delicious apples	6.9±3.1 bc	1258±607 b	0.49±0.35 b
A. ludens	Artificial diet[c]	18.3±3.4 bc	625,190±76,048 a	75.8±16.0 b
Diabrotica (Four spp.)	Artificial diet[d]	10.8±4.1 bc	7215±1008	59.9±24.5 b
ANOVA $F_{6,34}$ (P value)[e]	–	20.0 (< 0.001)	174 (< 0.001)	85.2 (< 0.001)

[a] Mean±SE; data shown are untransformed.

[b] Carolina Biological Supply 'Roach Food'.

[c] Corn and sugar-based artificial diet.

[d] Fructose and casein-based artificial diet.

[e] Statistics were performed on transformed data (see text for details).

of *O. fasciatus*, which did not yield detectable JA (Table 2). Benzoate and salicylate from the 7 diets showed statistical patterns similar to each other with the diets of *Anastrepha ludens* (Loew) and *P. americana*, respectively, having significantly greater concentrations than the other diets, which were similar to each other (Table 2).

Amounts of JA on a "per-gram" basis varied across insect species (Fig. 1a; ANOVA $F_{13,\ 80}$= 6.5, P<0.001). Eggs of the fly *Rhagoletis pomonella* (Walsh) had the highest levels of JA, significantly greater than concentrations in eggs of 10 of the other species (Tukey's HSD P< 0.05), whereas *A. ludens* eggs had a concentration that was similar to that of *R. pomonella* (Tukey's HSD P>0.05), but greater than that of the eggs of *P. americana*, *Tenodera aridifolia* Saussure, *Nezara viridula* L., and *O. fasciatus* (Tukey's HSD P<0.05). All remaining species were statistically similar to each other (Fig. 1a; Tukey's HSD P>0.05).

Benzoate levels also differed across species on a "per-gram" basis (Fig. 1b; ANOVA $F_{13,\ 80}$=15.8, P<0.001). Similar to our results for JA, eggs of *R. pomonella* had the greatest concentrations of BA, significantly greater than eggs of all the other species except *A. ludens* (Fig. 1b; Tukey's HSD P<0.05). Eggs of *A. ludens* had concentrations of BA that were higher than eggs of 5 species (Fig. 1b; *E. tiaratum*, *T. aridifolia*, *P. americana*, *O. fasciatus*, *N. viridula*; Tukey's HSD P<0.05). Eggs of *E. tiaratum*, *P. americana*, and *T. aridifolia* contained the lowest amounts of BA, which were statistically lower than the eggs of 6 other species (Fig. 1b, Tukey's HSD P<0.05). Eggs of the remaining species had similar levels of BA (Fig. 1b; Tukey's HSD P>0.05).

"Per-gram" levels of SA also varied among species (Fig. 1c; ANOVA $F_{13,\ 80}$=3.7, P= 0.001). Unfortunately, many species showed considerable variation among samples, resulting in statistical differences only between eggs of *R. pomonella* and the eggs of *E. tiaratum*, *T. aridifolia*, *Zootermopsis nevadensis* (Hagen), *N. viridula*, *O. fasciatus*, and the 4 species of *Diabrotica* (Fig. 1c, Tukey's HSD P<0.05). Levels of SA in eggs of the remaining species were intermediate and similar to levels found in eggs of all the other species (Tukey's HSD P>0.05).

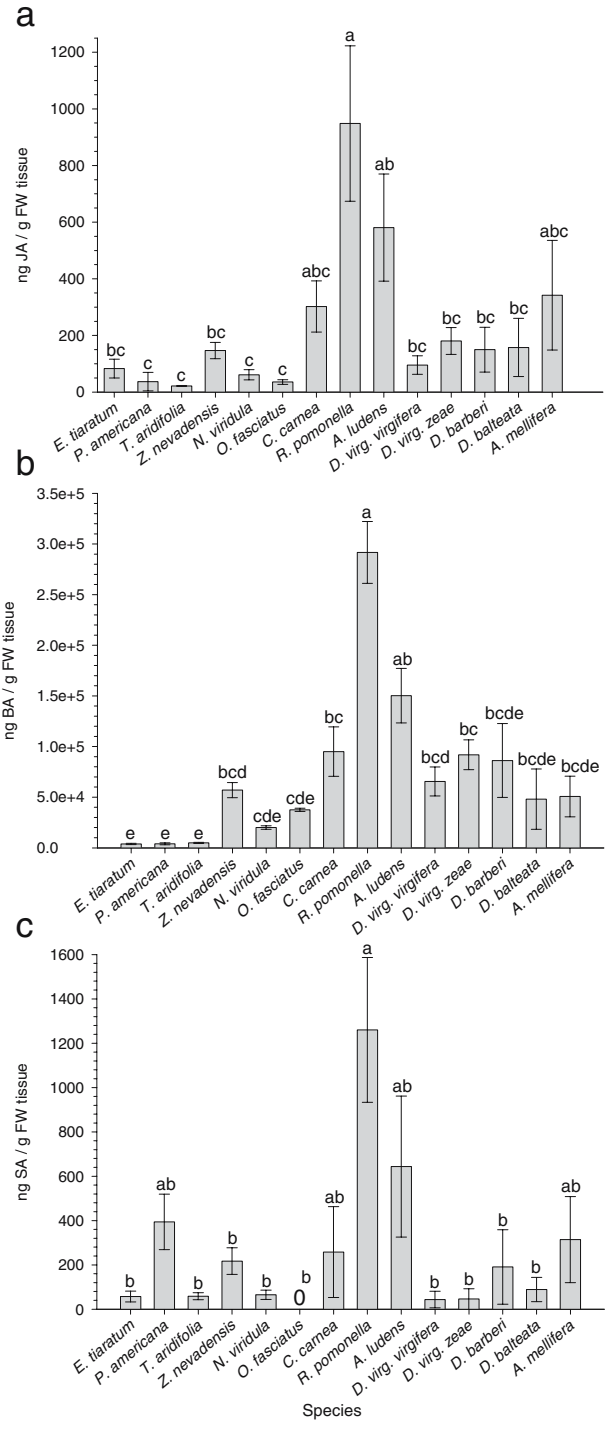


Fig. 1 (a) JA, (b) BA, and (c) SA per gram FW tissue recovered from eggs of 14 insect species. Bars marked with different letters are significantly different from each other (Tukey HSD $P<0.05$; see text for details on statistics)

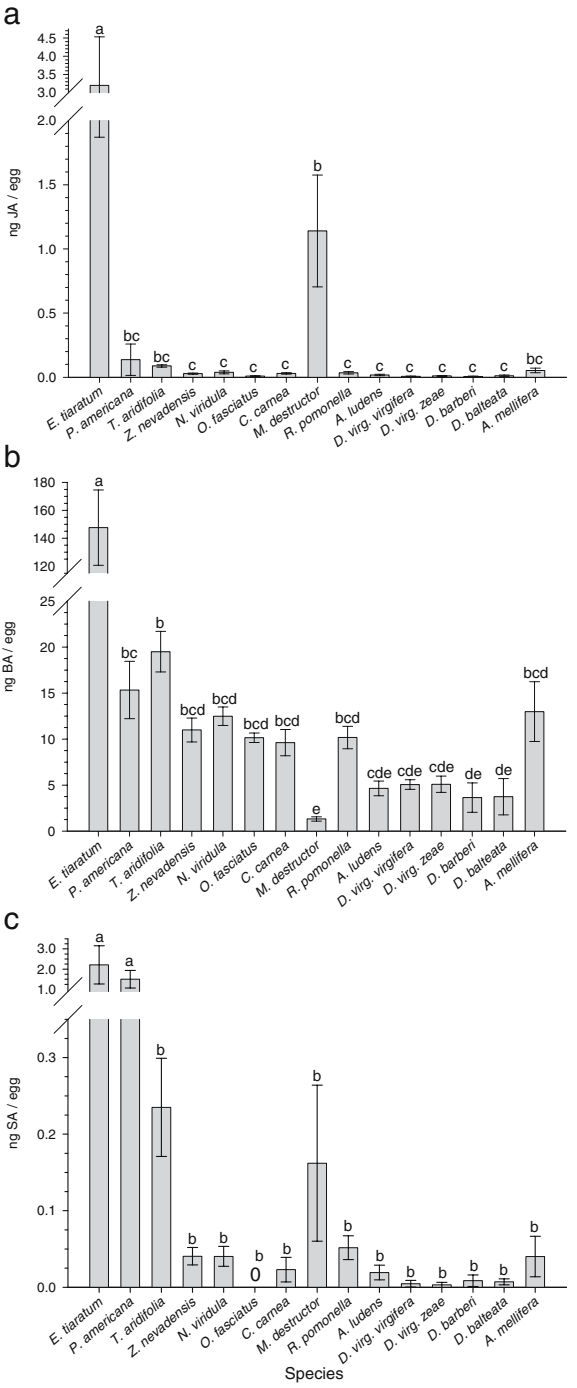


Fig. 2 (a) JA, (b) BA, and (c) SA per egg recovered from eggs of 15 insect species. Bars marked with different letters are significantly different from each other (Tukey HSD $P<0.05$; see text for details on statistics)

When considered on a "per-egg" basis, JA varied among eggs of different species (Fig. 2a, ANOVA $F_{14, 85}$=9.0, P<0.001). Eggs of *E. tiaratum* contained greater amounts of JA than all the other species, whereas eggs of *M. destructor* (Say) had the next highest amount, statistically greater than the levels detected in the eggs of all but 3 species (*P. americana*, *T. aridifolia*, and *Apis mellifera* L.; Tukey's HSD P>0.05). Benzoate also varied on a 'per-egg' basis, but the pattern was more complicated (Fig. 2b, ANOVA $F_{14, 85}$= 46.2, P<0.001). As with JA, eggs of *E. tiaratum* had a concentration that was greater than all other species (Tukey's HSD P<0.05). Eggs of the gall midge *M. destructor* had the lowest amounts of BA, whereas the remaining species were more or less intermediate (Fig. 2b). Salicylate also varied among species (Fig. 2c; ANOVA $F_{14, 85}$=10.2, P<0.001), showing a slightly different pattern than JA and BA. Eggs of *P. americana* and *E. tiaratum* had similar amounts of SA (Tukey's HSD P>0.05) that were greater than eggs of all other species (Tukey's HSD P<0.05).

Because *E. tiaratum* eggs, the largest of the species sampled, contained the greatest amounts of JA, BA, and SA, we suspected that amounts of these plant compounds might be related to egg size. Egg mass varied across species (ANOVA $F_{13, 80}$=1577, P<0.001) with *E. tiaratum* eggs being the heaviest and larger than eggs of all other species (38.1±0.9 mg/egg; Tukey's HSD P<0.05). Eggs of *P. americana* and *T. aridifolia* were the next largest and were similar in mass (4.1±0.4, 4.0±0.1 mg/egg, respectively; Tukey's HSD P>0.05) and larger than eggs of the remaining species (Tukey's HSD P<0.05). Eggs of the 11 remaining species (excluding *M. destructor*) had similar masses (ranging from 0.03 to 0.60 mg; Tukey's HSD P>0.05). Linear regression revealed that "per-gram" amounts of JA, BA, and SA were not statistically related to the mass of individual eggs (r^2≤0.07, P> 0.05); however, on a "per-egg" basis, JA, BA, and SA were positively related to mass of individual eggs, suggesting that larger eggs contain greater amounts of these plant-derived compounds (ng JA/egg vs. egg mass: r^2=0.58, P<0.001, best fit regression line Y=85.3X− 0.03; ng BA/egg: r^2=0.87, P<0.001, Y=3711X+7.01; ng SA/egg: r^2=0.45, P<0.001, Y= 59.2X+0.09). Therefore, we avoid any further consideration of "per-egg" data because it is undoubtedly confounded by egg mass.

Regression analysis also revealed that "per-gram" amounts of JA, BA, and SA were positively related to each other ("per-gram" statistics—ng SA/g vs. ng BA/g: r^2=0.49, P< 0.001, Y=0.004X−22.6; ng JA/g vs. ng BA/g: r^2=0.62, P<0.001, Y=0.003X−25.9; ng SA/g vs. ng JA/g: r^2=0.72, P<0.001, Y=1.11X+21.4). These relationships indicate that insect species that had high concentrations of one of these compounds in their eggs tended to have higher concentrations of the other two as well. Finally, linear regression revealed no statistical relationship between the amounts of JA, BA, and SA in eggs of different insect species and amounts of these compounds in their diets (r^2≤0.07, P>0.55), indicating that factors other than dietary concentrations influence the amounts of these compounds in eggs.

When we compared amounts of JA, BA, and SA in eggs of herbivores and predators, we found no statistical differences on a "per-gram" basis (262.7±52.3 and 162.0±63.2 ng JA/g for herbivores and predators, respectively, ANOVA $F_{1, 80}$=0.5, P=0.48; 84,368±11,738 and 49,949±18,910 ng BA/g, ANOVA $F_{1, 80}$=1.14, P=0.29; 319.9±67.8 and 158± 102.5 ng SA/g, ANOVA $F_{1, 80}$=0.76, P=0.39). When we divided the insect species into more specific functional groups based on nymphal or larval feeding habits, we found a consistent pattern for all three compounds: eggs of the fruit feeders had higher concentrations than eggs of species in other functional groups, which were statistically similar to each other (Fig. 3; ng JA/g: ANOVA $F_{3, 70}$=22.3, P<0.001; BA: ANOVA $F_{3, 70}$=24.9, P<0.001; SA: ANOVA $F_{3, 70}$=12.9, P<0.001).

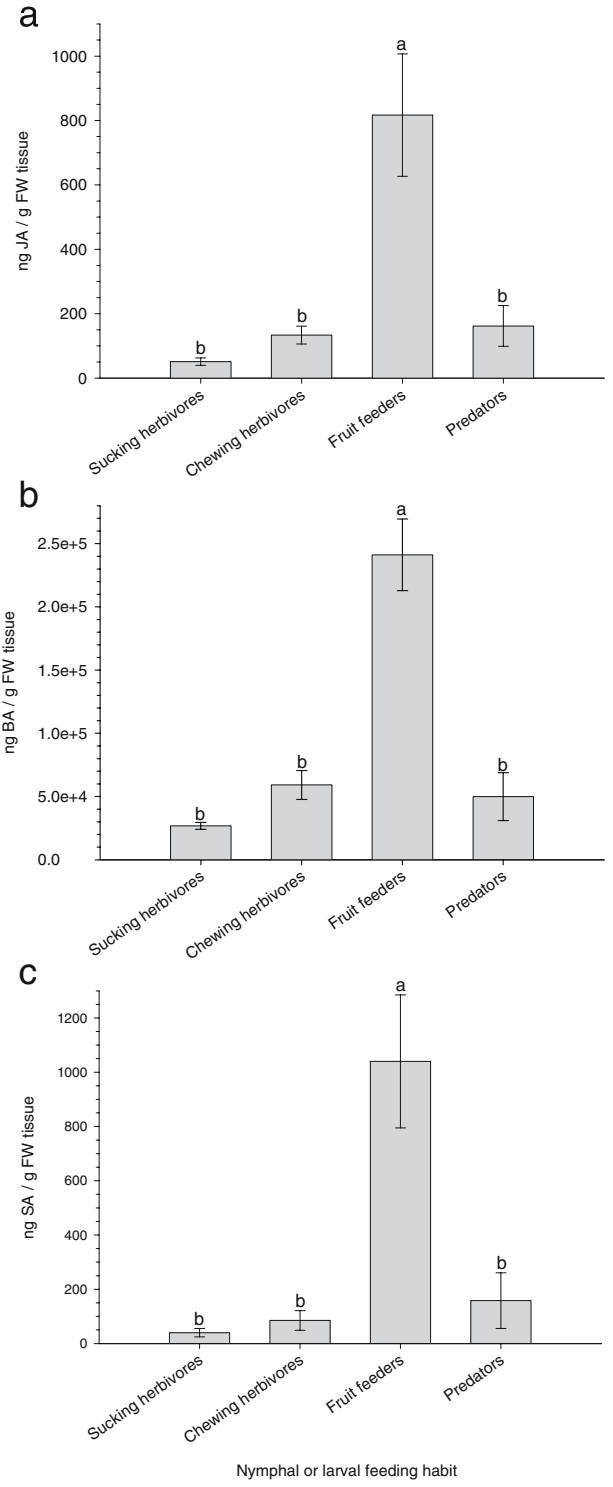


Fig. 3 (a) JA, (b) BA, and (c) SA per gram FW in eggs of four different functional groups based on nymphal or larval diets and represented by at least two species (see Table 1). Bars marked with different letters are significantly different from each other (Tukey HSD $P<0.05$; see text for details on statistics)

Discussion

All the diets we analyzed contained substantial amounts of BA (>1 μg/g diet FW), and all but one contained JA and SA. The exception was the sunflower-seed diet used to rear *O. fasciatus* (Table 2), whose eggs were the only ones analyzed that did not contain detectable amounts of SA (Fig. 1c), suggesting that SA (and JA and BA) in insect eggs is at least partially obtained from the nymphal or larval diets of their mothers. Thirteen of the species that we screened were either reared directly on plant material or plant-based artificial diets, and most of their diets contained all three compounds, sometimes in extremely high concentrations (Table 2). The two remaining species were predators: the lacewing *Chrysoperla carnea* was reared on a proprietary chicken egg-based artificial diet, and the plant-derived compounds could have originated in plant-based chicken feed; eggs of the mantid *T. aridifolia* were field collected, and JA, BA, and SA in mantid eggs could have originated from herbivores eaten as prey. Despite diets being the likely sources of plant-derived compounds, we found no clear relationship between amounts of JA, BA, or SA in the diet and "in-egg" concentrations. These findings agree with levels of JA detected in lepidopteran eggs and diet (Tooker and De Moraes, 2005) and indicate that other factors may play a role in determining the amounts of these compounds in eggs. Some evidence suggests that insect species may be capable of synthesizing these typically plant-derived compounds (Tooker and De Moraes, 2005). Similarly, we did not detect JA from sunflower seeds, but did detect JA in eggs of *O. fasciatus*, suggesting that this phytohormone may be produced by the insects themselves, although the possibility remains that JA was present in sunflower seeds, but was bound or otherwise undetectable by our methods.

The consistent presence of JA, BA, and SA in insect eggs across taxa suggests that these compounds may serve some function for eggs or developing insects. Moreover, species-specific concentrations suggest that insect species vary in their accumulation and/or use of these compounds. We have previously suggested that JA might perform an unidentified function in lepidopteran eggs, drawing on commonalities between plant and animal signaling systems and the structural similarity of JA and prostaglandins, which mediate a variety of functions in insects (Tooker and De Moraes, 2005). Our discovery of JA in eggs of eight more orders reinforces this idea. The JA concentrations for non-lepidopteran eggs that we report here span a lower range (20–950 ng JA/g FW) than those we reported for lepidopteran eggs (500 ng–600 μg JA/g FW; Tooker and De Moraes, 2005), but considerable overlap remains in the concentrations across different taxa. We are uncertain why lepidopteran eggs appear to contain more JA than eggs of other insect species, but perhaps, the physiological needs of caterpillars for JA are greater. Alternatively, chewing caterpillars may elicit a stronger JA response from plants causing JA to accumulate to a greater degree in their bodies and eggs.

Among non-lepidopteran species, eggs of the apple maggot *R. pomonella* contained the greatest "per-gram" concentrations of JA, BA, and SA (Fig. 1). Similarly, when we considered amounts across functional groupings, fruit feeders (comprising *R. pomonella* and *A. ludens*; Table 1) had the highest "per-gram" concentrations of JA, BA, and SA (Fig. 3). This finding was unexpected because most research on these three compounds has focused on "in-leaf" concentrations rather than levels in fruits, giving the impression that leaf concentrations are more significant; however, all three compounds appear common in fruit, including apples (Meyer et al., 1984; Peña-Cortés et al., 2005), the food for *R. pomonella*, and the fruit-mimicking diet used to rear *A. ludens* (Table 2). In fact, concentrations of JA in fruit of some plant species can greatly exceed concentrations found

in other plant tissues (Meyer et al., 1984). Jasmonate in fruit appears to play a role in ripening in addition to its role as a defensive phytohormone (Peña-Cortés et al., 2005). It should be noted, however, that whereas JA levels of *R. pomonella* eggs were the greatest among all species we screened (Fig. 1a), levels of JA in the diet were among the lowest we detected (Table 2), indicating that insects accumulate JA while they feed as larvae, and/or the species may be capable of producing JA on their own.

Benzoate and salicylate are also found in fruit (Table 2; Robertson and Kermode, 1981; Zuo et al., 2002). Salicylate is ingested by herbivores, providing a signal that warns of production of toxic plant compounds (Li et al., 2002). Moreover, when ingested by humans, BA- and SA-containing fruit can elevate levels of BA and SA in plasma (Zhang and Zuo, 2004). Perhaps frugivory also enriches these compounds in insects, favoring passive or active incorporation into eggs.

On a "per-egg" basis, the phasmid *E. tiaratum* had the highest levels of JA, BA, and SA, but this result likely reflected the size of *E. tiaratum* eggs (Fig. 2). In fact, significant linear relationships between amounts of JA, BA, and SA and the mass of individual eggs suggest that larger eggs tended to have high concentrations of these compounds, and this relationship likely explained any other "per-egg" patterns. It is worth noting, however, that we did not find a similar pattern for JA in lepidopteran eggs, whose masses spanned a much smaller range (0.05–3.0 mg/egg; unpublished data; Tooker and De Moraes, 2005). It is also worth noting that *E. tiaratum*, among the 15 insect species that we screened, has the mode of feeding that is most similar to leaf-feeding lepidopterans. However, on a "per-gram" basis, the concentrations of JA in *E. tiaratum* eggs (~85 ng JA/g) were considerably lower than the lowest concentration we found among lepidopteran eggs (~500 ng JA/g for *Manduca sexta*; Tooker and De Moraes, 2005). It seems likely that amounts of JA (or BA and SA) found in eggs of herbivores are a function of the insect species involved, the strength of the plant response to herbivory, and possibly, the insects' physiological usage of JA. The strength of the induced plant response appears to be influenced by host plant species, the tissue being attacked, and the mode of feeding, including content of the oral secretions of the insect species (Karban and Baldwin, 1997). Interactions among these various factors may explain why sucking and chewing herbivores had similar amounts of JA, BA, and SA, although chewing insects tend to elicit a stronger JA response from plants than sucking herbivores, which tend to elicit a stronger SA response (Walling, 2000).

As with JA (see above and Tooker and De Moraes, 2005), the presence of BA and SA in insect eggs is not easily explained. Both compounds have antimicrobial and antifungal properties that may confer some protection from pathogens (Krebs et al., 1983; Cowan, 1999). Moreover, SA can attenuate in both plants and animals the virulence of some pathogens (Prithiviraj et al., 2005), suggesting that insect eggs could gain similar protection from its presence. As phenolic compounds, BA and SA might protect eggs and developing insects by blocking harmful free radicals from oxidizing important biological molecules, including DNA (Zuo et al., 2002). Also, BA is a component of defensive secretions of some insect species (e.g., Classen and Dettner, 1983) and could conceivably deter egg predators.

At the same time, concentrations in eggs of SA and its precursor BA seem counterintuitive given their roles in initiating plant defenses against pathogens and some insect species (Walling, 2000; Delaney, 2005). Their presence in herbivore eggs that are laid on plants could provide a plausible mechanism for triggering an SA-mediated hypersensitive response in leaves (e.g., Shapiro and DeVay, 1987; Balbyshev and Lorenzen, 1997; Garza et al., 2001). Similarly, JA in insect eggs could play a role in volatile defensive

responses of plants against insect eggs (Meiners and Hilker, 2000; Tooker and De Moraes, 2005). However, it seems maladaptive for endogenous compounds of eggs (i.e., JA, BA, or SA) to facilitate such plant defense responses, and natural selection would likely not favor their inclusion under these circumstances in the absence of some other function (Tooker and De Moraes, 2005). Moreover, in preliminary work, we have not been able to detect measurable JA, BA, or SA after solvent washes of the exterior of eggs that are laid on plants (e.g., *M. destructor* eggs; unpublished data); however, different species and/or different extraction methods might yield a different result. Nevertheless, at least for hypersensitive responses after oviposition, no other triggering mechanisms appear to have been proposed, and possible roles for BA and SA in eggs warrant investigation.

In summary, the phytohormones JA and SA, and a precursor of salicylate, BA, appear common in insect eggs. The function of these in eggs is unclear, but they could serve signaling or defensive roles. It is also conceivable that they could play a role in triggering plant defenses after oviposition on plants. Further research is underway to clarify the exact role of the plant-derived compounds in insect eggs.

Acknowledgements We thank J. Saunders, A. Conrad, and C. Wagner for logistical support, E. Bogus for technical assistance, S. Colazza (University of Palermo), J. Lundgren, and C. Nielson (USDA, Brookings, SD, USA), R. Ruiz and O. Forrester (USDA, Edinburg, TX, USA), B. Thorne and N. Breisch (University of Maryland), S. Olsson and C. Smith (NY State Ag. Research Station, Geneva, NY, USA), and C. Delphia, M. Frazier, and O. Thompson (Penn State) for the eggs. The Hessian fly colony was established with the assistance of N. Bosque-Perez and D. Schotzko (University of Idaho). We thank M. Mescher and J. Runyon for comments on the manuscript. The project was supported by the USDA National Research Initiative (#2002-35302-12375), the David and Lucile Packard Foundation, and the Beckman Foundation.

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J Chem Ecol (2007) 33:345–351
DOI 10.1007/s10886-006-9217-6

Evaluation of Pheromones and a New Trap for Monitoring *Agriotes lineatus* and *Agriotes obscurus* in the Fraser Valley of British Columbia

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Received: 12 September 2005 / Revised: 16 September 2006 / Accepted: 11 October 2006 /
Published online: 27 December 2006
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Abstract A prototype ground-based pheromone trap design, baited with various pheromone lures, was field tested for effectiveness in trapping male *Agriotes obscurus* and *Agriotes lineatus* click beetles in British Columbia. Pheromone dispensers containing geranyl octanoate and geranyl hexanoate in a 1:1 ratio caught the greatest numbers of *A. obscurus*, whereas those containing geranyl octanoate and geranyl butanoate in a 9 or 10:1 ratio caught high numbers of *A. lineatus*. Some differences in *A. obscurus* attraction to traps were observed between dispensers according to the manufacturer and the number of dispensers deployed in traps. The trap design and optimal pheromone dispensers tested would be suitable for monitoring or surveying *A. obscurus* and *A. lineatus* populations in North America.

Keywords Elateridae · *Agriotes lineatus* · *Agriotes obscurus* · Semiochemicals · Pheromones · Agriculture · Fraser Valley · Surveys · Trapping

Introduction

The most economically important European wireworm species are in the genus *Agriotes* (Coleoptera: Elateridae) and include: *Agriotes brevis* Candeze; *Agriotes rufipalpis* Brulle; *Agriotes sordidus* Illiger; *Agriotes ustulatus* Schaller; *Agriotes sputator* L.; *Agriotes obscurus* L.; and *Agriotes lineatus* L. (Furlan et al., 2001a). Of these, *A. lineatus* and *A. obscurus* have been introduced to Atlantic Canada, British Columbia (BC), and Washington state in the USA (Eidt, 1953; Vernon and Päts, 1997; Vernon et al., 2001) whereas *A. sputator* has been reported in Nova Scotia (Eidt, 1953). These wireworms were probably introduced into North America at least a century ago with infested nursery stock

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and/or through the dumping of soil ballast in port areas (Eidt, 1953; Wilkinson, 1963). Where they have become established in Canada, *A. sputator, A. obscurus*, and *A. lineatus* have often become the dominant wireworm species and major economic pests of several crops (i.e., potatoes, corn, strawberries, cereal crops).

Surveys of European wireworm species in North America have traditionally relied upon habitat searches or devices such as pitfall traps for the click beetle stage (Vernon and Päts, 1997). Field surveys of *Agriotes* spp. wireworm stages are not usually considered due to the extensive labor and time required and difficulties in differentiating larvae of *A. obscurus* from *A. lineatus* (Wilkinson, 1963). However, the identification of sex pheromones for several *Agriotes* species (Yatsynin and Lebedeva, 1984; Kudryavtsev et al., 1993; Yatsynin et al., 1980, 1986, 1991, 1996; Borg-Karlson et al., 1988; Tóth et al., 2002a,b) offers a new tool for surveying and monitoring these economic elaterids. They were successfully used to survey the distribution of five species (*A. ustulatus, A. litigiosus, A. sputator, A. obscurus*, and *A. lineatus*) across the former Union of Soviet Socialist Republics (USSR) (Kudryavtsev et al., 1993), and surveys for eight species (*A. ustulatus, A. litigiosus, A. sputator, A. obscurus, A. lineatus, A. rufipalpis, A. sordidus*, and *A.brevis*) were carried out in several European countries between 1999 and 2001 (Furlan et al., 2001b). Studies are also underway in Europe to determine whether pheromone traps can be used as monitoring tools to determine the risk of wireworm attack at the field level (e.g., Furlan et al., 2001a).

The availability of sex pheromones for *A. obscurus, A. lineatus*, and *A. sputato*r would greatly enhance the scope and efficacy of surveys to determine the current geographic distribution of these species in North America. The objectives of this paper were to: (1) determine whether various pheromone lures produced by manufacturers in Hungary and Canada are effective in attracting *A. obscurus* and *A. lineatus* populations in BC; and (2) develop a ground-based pheromone trap for the general survey or in-field monitoring of *Agriotes* species in North America.

Methods and Materials

Pheromones and release devices *Agriotes lineatus* and *A. obscurus* females produce a sex pheromone composed of (*E*)-3,7-dimethyl-2,6-octadienyl [=geranyl] esters. *A. lineatus* females produce primarily geranyl octanoate (G8) with geranyl butanoate (G4) as a trace component, whereas *A. obscurus* females produce both geranyl octanoate (G8) and geranyl hexanoate (G6) (Borg-Karlson et al., 1988; Yatsynin et al., 1996; Tóth et al., 2003). Dispensers containing a 10:1 ratio of G8:G4 were effective in attracting male *A. lineatus* to flight traps in Europe (Tóth, unpublished data). A 1:1 ratio of G8:G6 caught male *A. obscurus* in Russia, but an analysis of female pheromone glands suggested that a 10:1 ratio might be more effective (Tóth, unpublished data). Consequently, various ratios of G8:G6 and G8:G4 were tested for attractiveness to *A. obscurus* and *A. lineatus*, respectively, in BC in 1999. In these trials, two types of dispensers were used. The first, from the pheromone Group (Budapest, Hungary), consisted of 0.7-ml polyethylene vials with lids (No. 730, Kartell Co., Italy). Active ingredients were added in 50% hexane solutions, with load volumes of 60 or 33 µl (1:1 and 10:1 G8:G6 ratios) for *A. obscurus* baits, and 33 µl (10:1 G8:G4 ratios) for *A. lineatus* baits. After the hexane was allowed to evaporate, each dispenser was closed and individually wrapped in aluminum foil sachets. Dispensers provided by Phero Tech Inc. (Delta, BC, Canada) consisted of 400-µl centrifuge tubes (Evergreen Scientific, Los Angeles, CA, USA) with load volumes of 50 µl. In 2000, the Phero Tech centrifuge tube dispensers (2 per trap) were compared to bubble cap dispensers

(Phero Tech Inc.) in *A. obscurus* traps, with load volumes of 175 μl in the bubble caps (1:1 ratio of G8:G6). All dispensers were stored separately at −20°C before deployment in the field.

Pheromone trap design A portable plastic exclusion trench (United States Patent # 5,926,999) developed to mass trap Colorado potato beetles (Hunt and Vernon, 2001) captured large numbers of *A. obscurus* in a fallowed field in Agassiz, BC in 1998 (R.S. Vernon, unpublished data). Using reconfigured components of the exclusion trench, a prototype ground-based trap was constructed to house pheromone dispensers and crawling *A. obscurus* and *A. lineatus* were collected (Fig. 1). The trap consists of two converging ramps (separated at the top by a 1-cm wide gap), which curved downwards at the apex to ensure that click beetles would slide into the enclosed cavity within. The ramps had 0.2-mm high elevated ridges, spaced 2 mm apart to assist beetles in climbing. A retractable flat tray, extending into the enclosed cavity from one of the two flat sides, was fitted with a 10× 15 cm yellow sticky card (Phero Tech Inc.) to catch and hold click beetles falling into the trap. A removable lid prevented water and larger beetles from entering the trap. The pheromone dispenser(s) was centered along the underside of the lid using double-sided tape, parallel to and 1 cm above the ramp opening.

Traps were installed so the floor of the trap was flush with the soil surface to afford a smooth transition to the ramps. For consistency, a 1-m square area around the traps was kept free of weeds, and traps were always oriented with ramps facing north–south. Traps

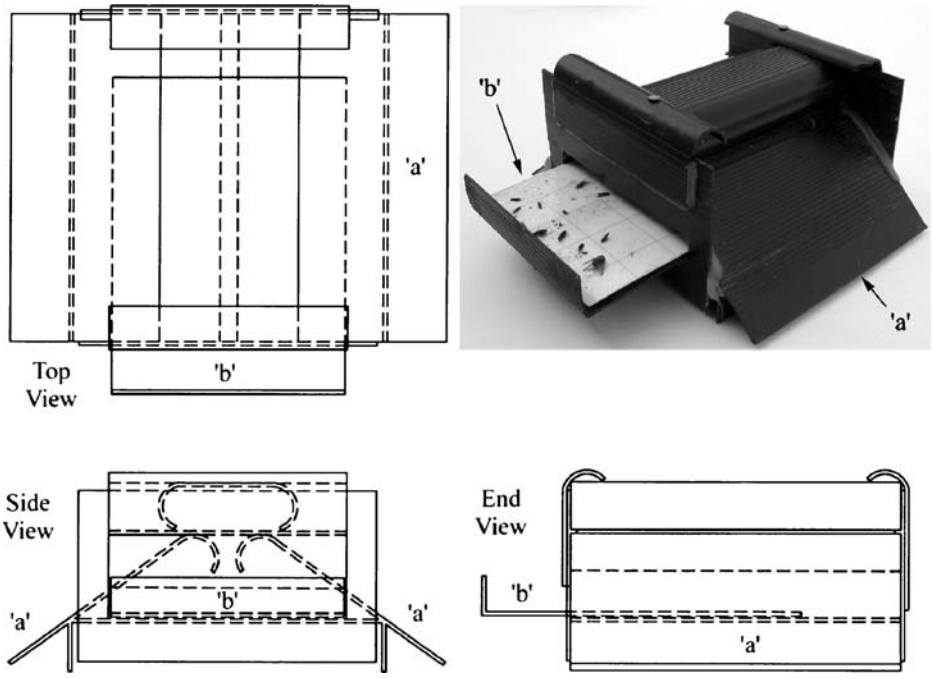


Fig. 1 Photograph and drawings of top, side, and end views of a ground-based pheromone trap developed for collecting crawling *Agriotes obscurus* and *A. lineatus* click beetles in 1999 and 2000. The opposing ramps (**a**) and partially extruded retractable tray with sticky trap (**b**) are indicated

were inspected by removing the tray and counting beetles on the sticky cards. At the end of each study, beetles on sticky cards were counted, as were any additional beetles that escaped the cards into the trap cavity. All elaterids captured were identified to species and subsamples of *A. obscurus* and *A. lineatus* identified to sex.

1999 Field Studies In 1999, experiments were conducted in fallow fields in Agassiz known to contain almost exclusively *A. obscurus* and in Delta in the lower Fraser valley of BC where *A. lineatus* was the predominant species (Vernon and Päts, 1997).

The first study conducted in Agassiz (5–13 May) tested the efficacy of prototype pheromone traps baited with 1:1 or 10:1 ratios of G8:G6), with dispensers produced in Hungary or Canada. The second (11–25 June) tested the efficacy of pheromone traps (1:1 ratio of G8: G6) baited with single (Hungary or Canada) or multiple (Canada) dispensers. Randomized complete block designs were used in both studies, with 5 m between traps in blocks and at least 20 m between blocks. Blocks were always arranged in consecutive linear arrays.

The first study conducted in Delta (10–21 May) tested prototype pheromone traps baited with dispensers (produced in Hungary or Canada) containing various ratios of G8:G4. The second (11–25 June) tested the efficacy of pheromone traps (9 or 10:1 ratios of G8:G4) baited with single (Hungary and Canada) or multiple (Canada) dispensers. Randomized complete block designs were used in both studies, with 5 m between traps in blocks and 10 m between blocks. Blocks were arranged in a linear array parallel to and about 10 m away from a grassy ditch alongside the fallowed field.

2000 Field Study A new 'bubble cap' dispenser developed by Phero Tech Inc. was tested for *A. obscurus* trapping efficacy in the prototype pheromone trap in Agassiz between 28 March and 1 May, 2000. This was compared with two Phero Tech centrifuge tube dispensers (all lures containing a 1:1 ratio of G8:G6), the most attractive to *A. obscurus* in 1999. The bubble cap and centrifuge tube dispensers were placed in the center of the sticky cards on the retractable trays inside the traps. The two traps were placed 5 m apart at four locations (20 m between replicates) along a 10-m wide fallowed strip of field adjacent to a grassy ditch.

Data Analysis The mean number of beetles collected in each trap were transformed $\left(y = (x + 0.5)^{1/2}\right)$ for analysis. Transformed means were compared using a single-factor analysis of variance (ANOVA) (proc GLM in SAS vers. 8.2, SAS Institute, Cary NC), followed by a Tukey–Kramer honestly significant difference test (α=0.05), or Duncan's multiple range test (trial 2, Table 1b).

Results

General observations With the more effective pheromone traps (Tables 1 and 2), adult click beetles of either *A. obscurus* (Agassiz studies) or *A. lineatus* (Delta studies) could be seen entering traps within minutes after trap installation. Subsamples of beetles that were sexed were predominantly male in the various studies, although some females (<1% of total) were also taken.

1999 Field Studies, Agassiz There were significant differences between various treatments in the first Agassiz study (ANOVA $F_{(4,24)}$=34.9, P<0.001). Traps baited with dispensers

Table 1 The effect of pheromone lure composition and quantity on *A. obscurus* attraction in a field in Agassiz, BC, in 1999

Treatment	Component Ratio			Lure Source[a]	Lures/Trap	*n*	Trap Days	Mean (± SEM) *A. obscurus*/trap[b]
	G8	G6	G4					
A: Agassiz, 5–13 May 1999								
1	0	0	0	Blank	n/a	5	8	1.2 (0.6)b
2	1	1	0	Hungary	1	5	8	150.0 (39.9)a
3	10	1	0	Hungary	1	5	8	16.4 (5.1)b
4	1	1	0	Canada	1	5	8	86.6 (20.3)a
5	10	1	0	Canada	1	5	8	17.6 (6.3)b
B: Agassiz, 11–25 June 1999								
1	0	0	0	Blank	n/a	4	14	1.3 (1.0)b
2	1	1	0	Hungary	1	3	14	71.3 (12.9)a
3	1	1	0	Canada	1	4	14	45.5 (8.8)a
4	1	1	0	Canada	2	4	14	113.5 (40.4)a
5	1	1	0	Canada	4	4	14	84.5 (23.9)a

[a] Hungary lures manufactured by The Pheromone Group, Budapest, Hungary. Canada lures manufactured by Phero Tech Inc., Delta, B.C.

[b] Means within trials followed by different letters are significantly different ($P<0.05$).

(Hungary and Canada manufacturers) containing a 1:1 ratio of G8:G6 were significantly more attractive to *A. obscurus* than the unbaited traps or those with dispensers containing a 10:1 ratio of G8:G6 (Table 1a). The 10:1 ratio of G8:G6, although catching some beetles, was not significantly different from the unbaited traps (Table 1a).

There were also differences between various treatments in the second Agassiz study (ANOVA $F_{(4,18)}=10.4$, $P=0.001$). Traps baited with one or more dispensers containing a

Table 2 The effect of pheromone lure composition and quantity on *A. lineatus* attraction in a field in delta, BC, IN 1999

Treatment	Component Ratio			Lure Source[a]	Lures/Trap	*n*	Trap Days	Mean (± SEM) *A. lineatus*/trap[b]
	G8	G6	G4					
A: Delta, 10–21 May 1999								
1	0	0	0	Blank	n/a	5	11	0.6 (0.4)b
2	10	0	1	Hungary	1	5	11	43.4 (2.0)a
3	9	0	1	Canada	1	5	11	64.0 (15.7)a
4	19	0	1	Canada	1	5	11	43.4 (8.5)a
5	19	0	1	Canada	2	5	11	62.2 (12.3)a
B: Delta, 11–25 June 1999								
1	0	0	0	Blank	n/a	4	14	0.3 (0.3)c
2	10	0	1	Hungary	1	4	14	47.3 (9.1)b
3	9	0	1	Canada	1	4	14	51.8 (7.1)b
4	9	0	1	Canada	2	4	14	77.5 (15.6)ab
5	9	0	1	Canada	4	4	14	109.0 (16.3)a

[a] Hungary lures manufactured by The Pheromone Group, Budapest, Hungary. Canada lures manufactured by Phero Tech Inc., Delta, B.C.

[b] Means within trials followed by different letters are significantly different ($P<0.05$).

1:1 ratio of G8:G6 were all more attractive to *A. obscurus* than the unbaited traps (Table 1b). However, there was no effect of the origin or number of lures (Table 1b).

2000 Field Studies, Agassiz Traps with bubble cap dispensers caught twice the number of *A. obscurus* as traps with two centrifuge tube dispensers (Canadian manufacturer) (mean= 134.0+30.9 vs. 64.3+15.1), although the difference was only marginally significant (ANOVA $F_{(1,7)}=7.3$, $P=0.07$).

1999 Field Studies, Delta In the first study, traps baited with the various ratios of G8:G4 were more attractive to *A. lineatus* than the unbaited traps (ANOVA $F_{(4,24)}=22.7$, $P<0.001$) (Table 2a). However, neither the ratios nor the origin of the lure affected trap efficacy (Table 2a). In the second study, all pheromone baited traps were more attractive to *A. lineatus* than the unbaited traps (ANOVA $F_{(4,19)}=32.6$, $P=0.001$). Increasing the number of dispensers improved beetle catch, with four Canadian dispensers catching significantly more beetles than traps with one dispenser from either Canada or Hungary (Table 2b).

Discussion

In the former USSR, it was shown that geographically isolated populations of *A. sputator, A. obscurus, A. lineatus*, and *A. ustulatus* may exhibit quantitative differences in major and minor pheromone components in pheromone gland extracts (Yatsynin et al., 1996). It was suggested that the presence of pheromone 'dialects' in *Agriotes* might lead to variability in response to pheromone traps in different geographic areas. In Canada, *A. lineatus* and *A. obscurus* probably became established in BC and certain Maritime provinces about a century ago (Eidt, 1953; Wilkinson, 1963), and at that time, established geographically isolated populations from their unknown European point(s) of origin. In the current study, *A. lineatus* responded optimally to a 9 or 10:1 ratio of G8:G4, and *A. obscurus*, to a 1:1 ratio of G8:G6, the same component ratios that have been successfully used to survey these species in several European countries (Furlan et al., 2001b; Tóth et al., 2003). These results suggest that these ratios would also be effective for surveying introduced populations of *A. lineatus* and *A. obscurus* in other parts of North America.

The current studies also show that trapping efficacy can be affected by the type and quantity of dispensers used. For example, traps baited with bubble cap dispensers caught more *A. obscurus* than those baited with two centrifuge tube dispensers (Canada). This may be due to differences in release rate, a parameter that was not measured in this study. In the case of *A. lineatus*, trap catch increased significantly when the number of dispensers was increased from one to four. However, as optimum trap efficacy is not a necessity for determining the presence or absence of these species, single centrifuge-style dispensers would be adequate and cost effective. Where greater efficacy is required, such as in a mass trapping program, either bubble cap dispensers (*A. obscurus* and probably *A. lineatus*), or multiple centrifuge tube dispensers (*A. lineatus*), could be used.

Pitfall traps were used in earlier *A. obscurus* and *A. lineatus* surveys in the lower Fraser valley of BC (Vernon and Päts, 1997). In a preliminary comparison at Agassiz in 1999, prototype pheromone traps (baited with dispensers from Hungary containing a 1:1 ratio of G8:G6) caught approximately 200 times more *A. obscurus* adults than pitfall traps (R.S. Vernon, unpublished data). Thus, as pheromone traps are far more effective than pitfall traps, they should be the method of choice in general surveys for *A. obscurus* and *A.*

lineatus (and possibly *A. sputator*) in North America. The potential use of these pheromone traps as mass trapping devices, or for estimating the risk of wireworm damage by *A. lineatus* and *A. obscurus*, is currently being investigated in fields in the lower Fraser valley of BC.

Acknowledgements This work was funded by the Matching Investment Initiative (project #A01560) of Agriculture and Agri-Food Canada, with assistance from Phero Tech Inc., the BC Potato Industry Development Committee, the BC Strawberry Growers Association, and the Lower Mainland Horticulture Improvement Association. We also thank Marcus Clodius for the analysis of data and Lorne Primeau for the figure preparation.

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J Chem Ecol (2007) 33:353–368
DOI 10.1007/s10886-006-9233-6

Differential Attraction of *Heliothis subflexa* Males to Synthetic Pheromone Lures in Eastern US and Western Mexico

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Received: 28 August 2006 / Accepted: 27 November 2006 /
Published online: 3 January 2007
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Abstract The mate attraction signal of *Heliothis subflexa* (Hs) females consists of a multicomponent sex pheromone blend. In this study, we assessed the intraspecific importance of three groups of compounds found in Hs pheromone glands: three acetate esters (Z7-16:OAc, Z9-16:OAc, and Z11-16:OAc), two 14-carbon aldehydes (14:Ald and Z9-14:Ald), and one 16-carbon alcohol (Z11-16:OH). Because the relative importance of pheromone components may vary in different regions, we conducted experiments in Eastern US (North Carolina) and Western Mexico (Jalisco). Our experiments in Eastern US showed that when the acetates were omitted from a 7-component blend in rubber septa, fewer males were caught in cone traps. Subsequent experiments conducted both in Eastern US and Western Mexico indicated that the addition of Z9-16:OAc alone does not increase attraction of male Hs, while Z11-16:OAc does. The Hs male response to Z7-16:OAc differed between the two regions. In Eastern US, significantly more males were attracted to a minimal three-component blend to which Z7-16:OAc was added, but this was not the case in Western Mexico. The two 14-carbon aldehydes also showed differential attraction between the two regions. 14:Ald and Z9-14:Ald appeared not to play any role in the sexual communication of Hs in Eastern US, but reduced trap catches in Western Mexico. The alcohol Z11-16:OH was tested in two concurrent dose–response studies with Hs males in Western Mexico, one using a minimal blend and one using a complete blend. The minimal three-component blend provided a more discriminating tool for delineating dose–response effects of Z11-16:OH than the seven-component blend. In the minimal blend, the optimal dose of Z11-16:OH was 1%, while in the complete blend similar numbers of males were caught when the alcohol ranged from 1 to 25%.

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Keywords Sexual communication · Pheromone · Pheromone component · Geographic variation · Acetate esters · 16-Carbon alcohol · 14-Carbon aldehydes · Dose–response · Minimal blend · Complete blend

Introduction

The evolution of premating signals is generally shaped by both intra- and interspecific selection. In moths, the predominant premating signals consist of multicomponent sex pheromone blends produced by females to which only conspecific males are attracted. To delineate the direction and magnitude of selection exerted on multicomponent pheromone blends, it is important to define the role of each constituent. The behavioral significance of pheromone compounds may vary geographically, and geographic variation in moth pheromone blends has been found in several species (e.g., Klun and Cooperators, 1975; Cardé et al., 1977; Guerin et al., 1984; Gemeno et al., 2000; McElfresh and Millar, 1999, 2001; Gries et al., 2001; El-Sayed et al., 2003). Such variation may be due to drift or to communication interference between closely related species in areas of sympatry. When closely related species with similar pheromone blends cooccur, kairomonal effects of specific compounds in a blend that inhibit attraction of closely related males may be important (Cardé et al., 1977; Löfstedt, 1990, 1993; Löfstedt et al., 1991; Butlin, 1995; Gries et al., 1996; Potting et al., 1999).

Heliothis subflexa (Guenée 1852) (Hs) (Lepidoptera: Noctuidae) is a New World specialist herbivore, and as it only feeds on species in the genus *Physalis* (McElvare 1941; Sheck and Gould, 1995, 1996) its occurrence is limited to habitats where these plants occur. In the US, *Physalis* species (e.g., ground cherry) are weedy plants, growing in disturbed habitats along roadsides and in agricultural fields. However, one species, tomatillo (*Physalis philadelphica* Lam.), is grown commercially in almost every state in Mexico. Thus, Hs appears to be patchily distributed in the US, while it is abundant in Mexico.

The intraspecific importance of each compound in Hs pheromone glands has not been firmly established (Table 1). Some of these compounds may merely be by-products of the pheromone biosynthetic pathway (Teal and Tumlinson, 1987; Jurenka and Roelofs, 1993; Jurenka, 1996, 2004) but most have not been tested systematically. For example, none of the three acetate esters has been tested separately, and research to date has generated contrasting results. Teal et al. (1981) found an increase in trap catch when all three acetates were added to a binary blend of Z11-16:Ald and Z9-16:Ald, whereas Vickers (2002) did not find a change in attraction in a wind tunnel when Z11-16:OAc was added to a three-component blend of Z11-16:Ald, Z9-16:Ald, and Z11-16:OH. We recently found a significant positive relationship between the relative percentage of acetates (sum of Z7-16:OAc, Z9-16:OAc, and Z11-16:OAc) in the pheromone glands of Hs females and the number of males attracted to them in the field (Groot et al., 2006). However, this relationship requires confirmation with synthetic blends, and it remains to be determined whether all three acetates are (equally) important.

At least three heliothine species are sympatric with Hs in parts of North America: the tobacco budworm *Heliothis virescens* (Hv) (Fabricius 1777), the corn earworm *Helicoverpa zea* (Hz) (Boddie), and *Heliothis phloxiphaga* (Hp) Grote & Robinson. Hv and Hz are both highly polyphagous species and are important agricultural pests (Laster, 1972; Sparks et al., 1979; Hartstack et al., 1979; Lopez et al., 1994; Parajulee et al., 2004). Hp is an innocuous oligophagous species feeding on asters and *Castilleja indivisa* (Texas

Table 1 Sex pheromone compounds of *H. subflexa* and their inferred role from previously published laboratory or field bioassays

	Gland Content	Volatile Blend	Tested
14:Ald	2, 7		2
Z9-14:Ald	2, 7		2
16:Ald	1, 2, 4, 7	4	1, 2, 3(−)
Z7/9-16:Ald[a]	1, 2, 4, 7	4	1(+), 2, 3, 5(+), 6(+)
Z11-16:Ald	1, 2, 4, 7	4	1(+), 2, 3, 5, 6(+)
16:OAc	2		2
Z7-16:OAc	1, 2, 4, 7	4	1(+), 2, 3
Z9-16:OAc	1, 2, 4, 7	4	1(+), 2, 3
Z11-16:OAc	1, 2, 4, 7	4	1(+), 2, 3, 5(0), 6(0)
Z9-16:OH	4	4	1(−), 3(0)
Z11-16:OH	1, 2, 4, 7	4	1(−), 2, 3, 5(+)

[a] Z7-16:Ald and Z9-16:Ald could not be separated on GC in some studies.

+: increased attraction, - : decreased attraction, 0 : did not affect attraction, no sign: only tested as part of a whole blend, thus a specific function cannot be inferred.

1 Teal et al., 1981 (tested different combinations of blends; found a significant decrease in attraction when 48% Z9-16:OH and 40% Z11-6:OH (relative to Z11-16:Ald) were added to a five-component blend); *2* Klun et al., 1982 (in the field no males were caught in traps with the two-component blend (Z11-16:Ald and Z9-16:Ald; these were tested in different ratios), while the 11-component blend caught males); *3* Heath et al., 1990 (varied the percentage of Z11-16:OH; attraction of Hs males was increased when 0.9–3.5% was released from the septa, but decreased when release rate was higher); *4* Heath et al., 1991; *5* Vickers and Baker, 1997; *6* Vickers, 2002; *7* Groot et al., 2004

paintbrush) (Eger et al., 1982), and it occurs only in the western part of North America (Forbes, 1954; Eger et al., 1982). Females of all four species produce overlapping multicomponent sex pheromone blends (Table 2), so that interspecific cross-attraction may occur at low frequencies (e.g., Chapin et al., 1997; Groot et al., unpublished research). Recently, we found that Hv can exert an impressive selection force on the production of acetate esters in Hs females (Groot et al., 2006).

In this study, we concentrated on three specific questions that emerge from studies on Hs pheromones: (a) What is the intraspecific role of each of the acetate esters found in the pheromone glands of Hs females? (b) Do 14:Ald and Z9-14:Ald, which are present in the Hs pheromone gland, play a role in the attraction of Hs males? (c) Are Hs males differentially attracted to blends with varying amounts of Z11-16:OH and does this response change when tested in a complete blend versus a minimal blend?

In the context of evolution of pheromone blend variants, we hypothesized that the value of pheromone components may differ regionally in response to interspecific interactions with species that have overlapping chemical communication channels. Thus, we reasoned that if the importance of the three acetate esters, 14:Ald, Z9-14:Ald, and Z11-16:OH, in the pheromone communication of Hs depends on the abundance of other heliothine moths and the probability of communication interference, then the following regional differences may be predicted. In areas where Hz and Hv are abundant (North America), Hs males are more likely to be attracted to blends containing the acetates and/or Z11-16:OH because their presence is indicative of, and unique to, the volatile blend of Hs females (Heath et al., 1991). Moreover, both the acetates and the alcohol will be important to antagonize attraction of Hv and Hz males (Hartstack et al., 1980; Vetter and Baker, 1983; Vickers and Baker, 1997; Fadamiro and Baker, 1997; Quero and Baker, 1999). In contrast, Hs males

Table 2 Behaviorally tested and confirmed sex pheromone components of four heliothine species in North America

Compounds found in Hs Glands	*H. subflexa*[1]	*H. virescens*[2]	*Helicoverpa zea*[3]	*H. phloxiphaga*[4]
14:Ald				
Z9-14:Ald		+++		
16:Ald			++	
Z7-16:Ald			++	
Z9-16:Ald	+++		++	++
Z11-16:Ald	++++	++++	++++	++++
16:OAc				
Z7-16:OAc				
Z9-16:OAc				
Z11-16:OAc		−	−	
Z9-16:OH	0			
Z11-16:OH	+++	−	−	+++

[1] Teal et al. (1981); Klun et al. (1982); Heath et al. (1990, 1991); Vickers and Baker (1997); Vickers (2002)

[2] Roelofs et al. (1974); Klun et al. (1979, 1980a); Tumlinson et al. (1975, 1982); Pope et al. (1982); Vetter and Baker (1983)

[3] Klun et al. (1979, 1980b); Vetter and Baker (1984); Pope et al. (1984); Vickers et al. (1991); Fadamiro and Baker (1997); Fadamiro et al. (1999); Quero and Baker (1999); Quero et al. (2001)

[4] Raina et al. (1986)

+: Increased attraction, −: decreased attraction, 0: did not affect attraction, ++++: primary sex pheromone component, +++: secondary sex pheromone component, ++ minor sex pheromone component

should be less attracted to blends containing 14:Ald and Z9-14:Ald in areas with large Hv populations because they risk the possibility of being attracted to Hv females that produce these two compounds in higher amounts (Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Klun et al., 1979, 1980a; Teal et al., 1986).

Methods and Materials

Pheromone Lures Pheromone components were obtained from *PHERO*BANK (Wageningen, The Netherlands), Shin-Etsu Chemical (Tokyo, Japan) and Bedoukian Research (Danbury, CT, USA). The following synthetic compounds were tested (% purity by GC indicated): Z11-16:Ald (98.0%; 16:Ald was the largest contaminant at 0.20%), 14:Ald (>98%), Z9-14:Ald (95.5%), 16:Ald (>98%), Z7-16:Ald (98.5%), Z9-16:Ald (96.6%), Z7-16:OAc (97.8%), Z9-16:OAc (99.2%), Z11-16:OAc (95%), and Z11-16:OH (95%).

Compounds were dissolved (*w*/*v*) in dichloromethane (2003) or *n*-hexane (2004 and 2005). Treatment blends were made relative to the primary component, Z11-16:Ald, which was loaded onto rubber septa at 100 μg (2003) or 300 μg (2004 and 2005). The ratios of all other compounds were based on Heath et al. (1991), who determined the release ratios of each of the *H. subflexa* pheromone components from septa similar to those used in this study. BHT was added at 1% (w/*v*) to all solutions, and ratios were confirmed by GC-FID on a DB-WAXETR (extended temperature range) column (30 m×0.25 mm×0.5 μm), which adequately separated Z7-16:Ald and Z9-16:Ald.

Rubber septa (#1780J07, Thomas Scientific, Philadelphia, PA, USA) were first ultrasonicated for 6 hr in hexane, then extracted ×3 with hexane, and air-dried in a fume

hood for at least 48 hr before loading. Each septum was first loaded with 100 µl of one of the treatment solutions, air-dried, then 100 µl of solvent were added to the septum well, air-dried again, and then stored in glass vials at −20°C until used. The septa were hung on a metal paper clip attached to a metal wire with a binder clip at each end, so that they could be positioned in the center of the trap opening without touching any trap surface. All septa were replaced at the same time, every 1–2 wk.

Field Sites Three different field sites were used in Eastern US: the North Carolina State University field station in Clayton, NC (35° 39′ 58″ N, 78° 30′ 36″ W), a commercial soybean field site in Smithfield, NC, USA (35° 30′ 42″ N, 78° 21′ 08″ W, ±20 km south of Clayton), and a commercial tomatillo field site in Oxford, NC (36° 13′ 12″ N, 78° 41′ 16″ W), ~100 km north of Clayton). In Western Mexico, commercial tomatillo fields in the state of Jalisco at Chamela (19° 31′ 49″ N, 105° 03′ 47″ W), Hidalgo (19° 21′ 37″ N, 104° 52′ 28″ W, ~25 km south of Chamela), and Autlán (19° 46′ 03″ N, 104° 20′ 59″ W, ~100 km northeast of Chamela) were used. Table 3 provides a summary of the site(s) used for each experiment.

Species-Specificity To determine species-specificity of the Hs and Hv blends, the following treatments were compared concurrently (Table 4): (1) the complete 7-component Hs blend (HsC treatment), based on Heath et al. (1990, 1991), (2) HsC minus the three acetate esters (HsC minAcs treatment), (3) the complete 7-component Hv blend (HvC treatment), based on Teal et al. (1986) and Heath et al. (1991), (4) HvC plus the three acetate esters (HvC+ Acs treatment), and (5) the control, in which rubber septa were loaded only with solvent and 1% BHT. For all septum loadings, the ratios of all compounds, except Z11-16:OH, were rounded off to the nearest 5% from the ratios reported by Heath et al. (1990). This experiment was conducted twice in both 2003 and 2004 (Table 3). The cotton and soybean

Table 3 Locations and dates of different experiments conducted on the sex pheromones of *H. subflexa* in Eastern US and Western Mexico

Test	Eastern US	Western Mexico (all *Physalis*)
Species-specificity	Clayton, NC: 28 Jul–18 Sep, 2003 (cotton) 30 Jul–31 Aug, 2004 (cotton) 16 Sep–6 Oct, 2004 (soybean) Smithfield, NC: 30 Sep–21 Oct, 2003 (soybean)	
Role of each acetate	Clayton, NC: 27 Jul–29 Aug, 2005 (cotton, *Physalis*)	Autlan, Jalisco 18 Oct–25 Oct, 2005 Hidalgo, Jalisco 17 Nov–23 Nov, 2005 21 Nov–24 Nov, 2005
Role of 14:Ald and Z9-14:Ald	Clayton, NC 1 Jul–22 Jul, 2005 (*Physalis*) Oxford, NC 12 Sep–28 Sep, 2005 (*Physalis*)	Hidalgo, Jalisco 29 Oct–3 Nov, 2005
Dose–response of Z11-16:OH		Hidalgo, Jalisco 3 Nov–8 Nov, 2005 Chamela, Jalisco 3 Nov–8 Nov, 2005

Table 4 Attraction of *H. subflexa* males in Eastern us to different synthetic blends to determine species-specificity

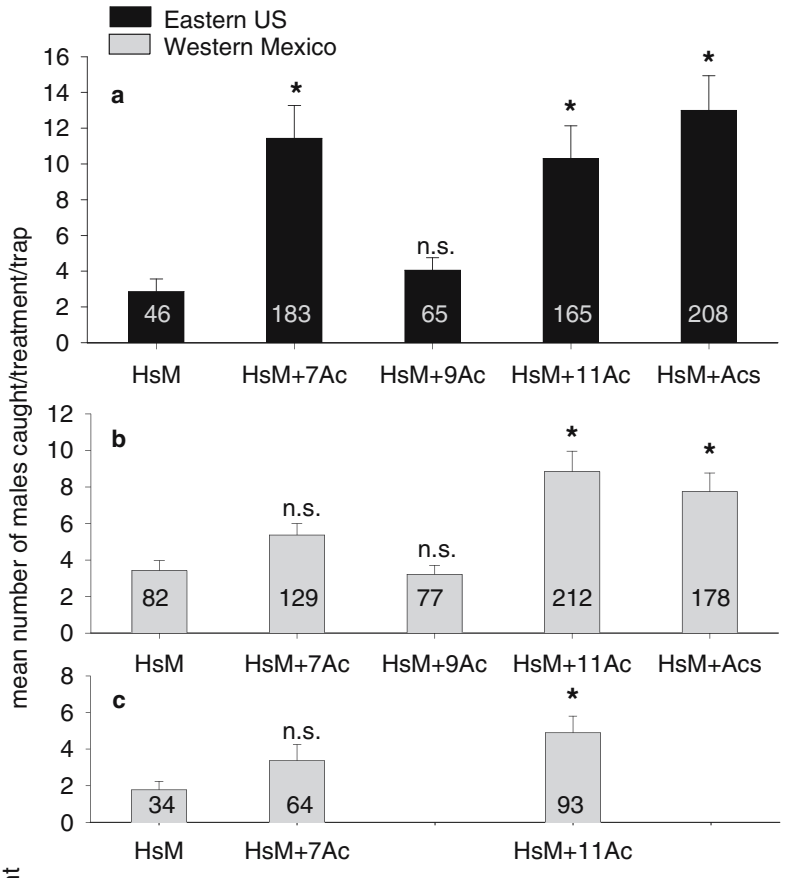
Treatment Name	Treatment Composition										Hs Males Caught Per Trap (mean± SEM)		Total No. of Males Caught
	14: Ald	Z9-14: Ald	16: Ald	Z7-16: Ald	Z9-16: Ald	Z11-16: Ald	Z7-16: OAc	Z9-16: OAc	Z11-16: OAc	Z11-16: OH			
HsC			5		50	100	5	10	20	10	0.49±0.088	a	45
HsC minAcs			5		50	100				10	0.29±0.083	b	26
HvC	5	5	10	2	2	100				1	0	–	0
HvC+Acs	5	5	10	2	2	100	5	10	20	1	0.022±0.022	c	2
Control											0.022±0.015	–	2

The experiment was conducted twice in 2003 and twice in 2004 (Table 3). Statistical analysis showed a significant date effect but not a significant year effect; therefore, we combined all data for further analysis. The treatment names are explained in the text, while their composition is shown in the table (the numbers refer to the percentages of each compound relative to Z11-16:Ald). Different letters indicate significant differences in mean number of males caught (ANOVA, Tukey's adjustment for multiple comparisons, $P < 0.05$). HvC treatment was not included in the analysis because no Hs males were caught in traps baited with this blend. The control treatment was omitted from the analysis because the specific question was whether the attraction of the treatments differed from each other.

fields in Clayton were about 100 m apart, and about 150 m from a field in which *Physalis* species had been planted to attract and generate an Hs population. Hartstack cloth traps (Hartstack et al., 1979) were arranged 15–20 m apart in a completely randomized Latin square design consisting of three replicates (rows), for a total of 15 traps in each field. These traps consist of a conical base leading to a removable top in which males are trapped. The five treatments were rotated every 3–4 d, the tops were replaced and males caught in the traps during 3–4 nights were sorted by species under a microscope.

Role of Each of the Acetate Esters To specifically determine whether Z7-16:OAc, Z9-16:OAc, or Z11-16:OAc are important in the attraction of conspecific males, an experiment consisting of five treatments was conducted in 2005 (see Fig. 1): a treatment consisting of the primary component Z11-16:Ald and the two secondary sex pheromone components Z9-16:Ald and Z11-16:OH, considered the Hs minimal blend (HsM) (based on Vickers, 2002); three treatments in which each of the three acetate esters was added to HsM (HsM+7Ac, HsM+9Ac, HsM+11Ac), and one treatment in which all three acetates were added (HsM +Acs). This experiment was conducted both in Eastern US and Western Mexico to investigate possible geographic differences in response to the different treatments. In Eastern US, the experiment was conducted in a field planted with *P. angulata*, *P. pubescens*, *P. cordata*, and *P. heterophylla* at Clayton (Table 3). Two replicates per treatment were used and the treatments were rotated every 2–3 d. In Autlán, Western Mexico, the experiment

Fig. 1 The effect of adding three acetate esters, separately and in combination, to the minimal blend on the attractiveness of lures to male *Heliothis subflexa* in Eastern United States and Mexico. A significant increase in number of Hs males caught is indicated by asterisks in a, b, and c. Asterisks in d indicate a significant difference between Eastern US and Western Mexico in the elevated attraction of Hs males compared to the minimal blend (i.e., the addition of Z7-16:OAc and the three acetate esters resulted in a significantly higher increase in attraction in Eastern US than in Western Mexico). ns represents "not significant", the error bars show +SEM, and numbers within bars indicate total number of Hs males caught. Each blend is represented at the bottom of the figure by percentages of each compound relative to Z11-16:Ald



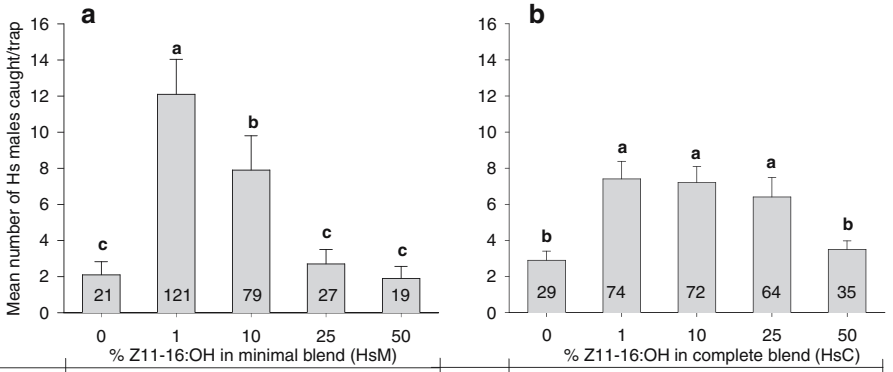
	HsM	HsM+7Ac	HsM+9Ac	HsM+11Ac	HsM+Acs
14:Ald					
Z9-14:Ald					
16:Ald					
Z7-16:Ald					
Z9-16:Ald	50	50	50	50	50
Z11-16:Ald	100	100	100	100	100
Z7-16:OAc		5			5
Z9-16:OAc			10		10
Z11-16:OAc				20	20
Z11-16:OH	10	10	10	10	10

consisted of three replicates per treatment, rotating the treatments every day. In addition to the complete experiment, an additional experiment was conducted in Hidalgo, Western Mexico, where HsM+7Ac and HsM+11Ac were compared with HsM. This experiment was replicated twice (Table 3), once with two replicates per treatment and once with three replicates per treatment, rotated every day.

Role of 14:Ald and Z9-14:Ald To determine whether 14:Ald and Z9-14:Ald are important in the attraction of Hs males, an experiment was conducted in 2005 comparing HsC and HsC with 14:Ald and Z9-14:Ald added (HsC+14Alds). This experiment was conducted at Clayton, using five replicates per treatment that were rotated every 3–4 d, eight times in total, and in Oxford, where both treatments were rotated every 2 d. This experiment was also conducted in Hidalgo, Western Mexico, where both treatments were replicated four times and rotated every 1–2 d, four times in total.

Dose–Response Test of Z11-16:OH To determine the effect of Z11-16:OH at different doses, two dose–response experiments were conducted concurrently. In Chamela, Z11-16:OH was varied from 0 to 50% in the minimal blend (HsM), while in Hidalgo the same doses were tested when added to the complete blend, HsC (see Fig. 2). In both experiments, two replicates per treatment were used and all treatments were rotated daily over 5 d.

Statistical Analyses The number of males caught per treatment in each experiment were square-root transformed, when needed, to normalize the variance. To determine the role of each of the acetate esters, a mixed model ANOVA was carried out using proc GLM in SAS



14:Ald										
Z9-14:Ald										
16:Ald						5	5	5	5	5
Z7-16:Ald										
Z9-16:Ald	50	50	50	50	50	50	50	50	50	50
Z11-16:Ald	100	100	100	100	100	100	100	100	100	100
Z7-16:OAc						5	5	5	5	5
Z9-16:OAc						10	10	10	10	10
Z11-16:OAc						20	20	20	20	20
Z11-16:OH	0	1	10	25	50	0	1	10	25	50

Fig. 2 Dose–response experiment with Z11-16:OH in Hidalgo and Chamela, Western Mexico. a: Z11-16:OH added to the minimal three-component blend, b: Z11-16:OH added to the complete seven-component blend. Different letters within each graph indicate significant differences in mean number of males caught and the error bars show +SEM. Numbers within bars indicate total number of males caught. Each blend is represented by percentages of each compound relative to Z11-16:Ald

9.1 with "treatment" and "day" as fixed effects, and "replicate" and "replicate by treatment" as random effects. To determine whether the addition of each of the acetate esters to HsM increased attraction, the number of Hs males captured in each treatment was compared to the number attracted to HsM alone, using Dunnett's procedure with "replicate by treatment" as the error term.

Geographic differences in attraction between Eastern US and Western Mexico to the acetate treatments were compared after normalizing the trap catches relative to those in HsM baited traps for each region. Subsequently, the increase in attraction to each blend in Eastern US was compared to the increase in attraction to each blend in Western Mexico, using proc MIXED in SAS 9.1, where "treatment", "region", and "day" were considered fixed factors, and "replicate", "trap position" and "treatment by trap position" per site were considered random effects.

Differences in attraction of (a) the species-specific blends, (b) the HsC and HsC+14Alds, and (c) the alcohol treatments, were compared using ANOVA (proc MIXED in SAS 9.1). Within each experiment, the numbers of males attracted to the different treatments were compared, where "treatment" and "day" were considered fixed factors, and "replicate" and "trap position" were regarded as random. The treatment means were then separated using Tukey's adjustment for multiple comparisons.

Results

Species-Specificity The HsC blend attracted significantly more Hs males than the HsC minAcs blend, while the Hv blend, with or without the acetates, did not differ from the control (Table 4).

Role of Each of the Acetate Esters Significantly more Hs males were caught in traps with HsM+11Ac and HsM+Acs than in traps with HsM alone in both regions, while the addition Z9-16:OAc did not increase attraction (Fig. 1a, b). In the case of HsM+7Ac, trap catches only increased in Eastern US (Fig. 1a). The lack of attractiveness of Z7-16:OAc for Mexican Hs males was confirmed in independent trials (Fig. 1b, c).

After normalizing the relative trap catches to that of the minimal blend, there were significant effects of region, treatment, day and their interactions (see Table 5), and there were contrasts between Eastern US and Western Mexico when a) HsM+7Ac, and b) HsM+Acs were compared (Fig. 1d). In both cases, more Hs males were caught in Eastern US than in Western Mexico.

Table 5 Type 3 tests of fixed effects when *H. subflexa* trap catches of the five acetate treatments were compared between Eastern US and Western Mexico

Effect	Degrees of Freedom	*F* value	Pr>*F*
Region	1	8.27	0.027
Treatment	4	33.26	<0.001
Region×treatment	4	5.69	<0.001
Day	7	5.92	<0.001
Region×day	7	10.13	<0.001
Treatment×day	28	1.68	0.031
Region×treatment×day	28	1.50	0.072

Role of 14:Ald and Z9-14:Ald In Eastern US, the addition of 14:Ald and Z9-14:Ald did not increase the attractiveness of the HsC blend (Table 6). However, in Western Mexico, more Hs males were caught in traps baited with the HsC (N=166) than those with HsC+14Alds (n=110, P=0.014).

Dose–Response of Z11-16:OH Trap catches of Hs males showed a nonmonotonic function in relation to increasing Z11-16:OH doses in both HsC and HsM blends, represented by parabolic, or inverted U-shaped curves (Fig. 2). Omission of the alcohol, as well as high doses of it, suppressed trap catch, whereas intermediate doses increased trap catch. However, the specific effective doses depended upon whether the alcohol was in the minimal or complete blend. Similar numbers of Hs males were caught with 1 to 25% Z11-16:OH in HsC, whereas significantly more males were only caught in traps baited with HsM and 1% Z11-16:OH (Fig. 2).

During these experiments, we also deployed pheromone traps in both Eastern US and Western Mexico to estimate the relative abundance of *H virescens*. A comparison of traps baited HvC and HsC in Eastern US showed that there were similar, or more, Hv males as Hs males caught in all 3 yr (Fig. 3). In contrast, in Western Mexico only three Hv males were captured in fields of commercially grown *Physalis*, compared with 785 Hs males.

Discussion

A thorough evaluation of the contribution to mating success of each pheromone compound found in the glands of *H. subflexa* females is critical to our goal of understanding the evolutionary diversification of heliothine moth pheromone signals (Groot et al., 2004, 2006; Sheck et al., 2006) In this study, we assessed the importance of several compounds whose roles in Hs sexual communication were equivocal.

The importance of pheromone compounds may differ geographically when their behavioral significance partly depends on the presence and/or abundance of closely related species that are attracted to similar pheromone blends. Of the common heliothines in

Table 6 Effects of 14:Ald and Z9-14:Ald on *H. subflexa* trap catches in Eastern US and Western Mexico

Treatment Name	Treatment Composition										Hs Males Caught Per Trap (mean±SEM)	
	14:Ald	Z9-14:Ald	16:Ald	Z7-16:Ald	Z9-16:Ald	Z11-16:Ald	Z7-16:OAc	Z9-16:OAc	Z11-16:OAc	Z11-16:OH	Eastern US	Western Mexico
HsC			5		50	100	5	10	20	10	3.63±0.46 a (N=232)	10.38±1.47 a (N=166)
HsC+14Alds	2	2	5		50	100	5	10	20	10	3.48±0.45 a (N=230)	6.88±0.78 b (N=110)

Different letters within region indicate significant differences in mean number of males caught (P<0.05). Each blend is represented by percentages of each compound (w/v) relative to Z11-16:Ald. HsC is the complete seven-component blend, and HsC+14Alds represents the addition of 14:Ald and Z9-14:Ald to the complete blend.

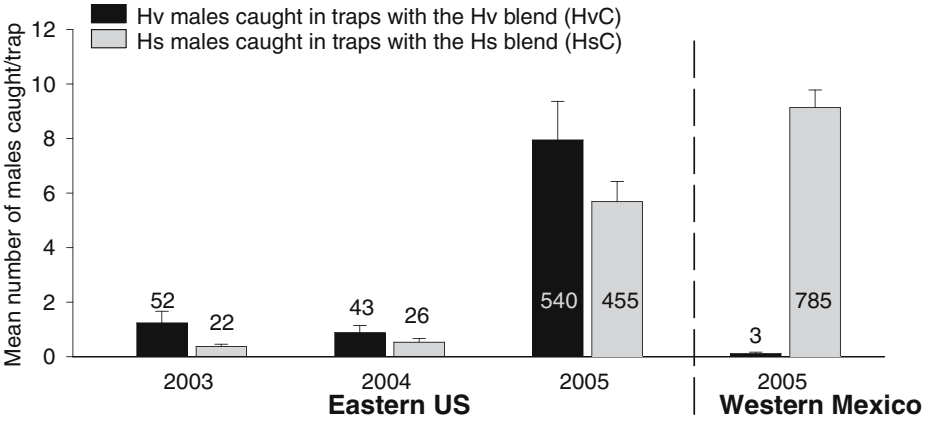


Fig. 3 Mean number of Hv and Hs males caught in traps with their respective pheromone lure during the trapping experiments conducted in Eastern US (years 2003–2005) and Western Mexico (2005). Numbers above/in bars indicate total number of males caught and the error bars show +SEM

Eastern US, Hv is the most closely related species to Hs (Mitter et al., 1993). Females of both species elicit interspecific precopulatory behavior from males in close proximity (Klun et al., 1982), and they can be hybridized in the lab, producing sterile male, and less fit fertile female, offspring (Laster, 1972; Sheck and Gould, 1995). However, males of these two species are rarely attracted to heterospecific females in the field (Groot et al., 2006). Trap catches in pheromone traps baited with the complete Hv blend confirm our supposition that Hv is much less abundant in Western Mexico than in Eastern US. Therefore, the chance of communication interference—that is, the likelihood of Hs females attracting heterospecific Hv males—is much greater in Eastern US than in Western Mexico.

Role of the Acetate Esters The species-specificity experiments conducted in Eastern US showed that one or more of the three acetate esters contribute to greater attraction of Hs males (Table 4). This experiment supports our finding that backcross Hs females who produce a very low amount of the acetate esters (<5% relative to all compounds present in the gland) were less attractive to Hs males than those with higher amounts (Groot et al., 2006). Notably, the context in which the acetates are presented is important as adding the three acetates to an HvC blend failed to attract more Hs males.

Of the three acetate esters emitted by calling Hs females, Z9-16:OAc does not appear to play a role in the long distance attraction of conspecific males in either Eastern US or Western Mexico. Hence, the presence of this compound in the Hs pheromone gland may just be a by-product of the biosynthetic pathway that produces the critical secondary pheromone component Z9-16:Ald, or may serve an antagonistic function to suppress attraction of other species, as has been found for Z11-16:OAc.

The addition of Z7-16:OAc to the HsM blend increased captures of Hs males in Eastern United States, whereas in Western Mexico HsM and HsM+7Ac gave similar results (Fig. 1). This geographic difference was even more evident when normalized trap catches were contrasted between the two regions (Fig. 1d).

The HsM+Acs lure was more attractive than HsM in both sites (Fig. 1a, b) but the addition of all three acetate esters resulted in a greater increase in Eastern US than in Western Mexico (Fig. 1d). This finding confirmed our prediction, based on our earlier

observations (Groot et al., 2006), that in Eastern US, where Hv and Hz are abundant, the acetates would not only function to enhance intraspecific communication but also to prevent the attraction of heterospecific males. Where the probability of communication interference with Hv and Hz is low, as in Western Mexico, selection pressure to produce acetates may be attenuated and Hs males may be more finely tuned to the other pheromone components (Z11-16:Ald, Z9-16:Ald, and Z11-16:OH). It will be interesting to investigate the interspecific interaction between Hs and Hp because their pheromone blends are more similar to each other than to Hv or Hz (Table 2), and one or more of these acetate esters might serve to reduce interspecific attraction of Hp males. However, Hp is an innocuous nonpest species and its population densities may be relatively low in areas where tomatillo is a major agricultural crop. In Western Mexico, no Hp males were caught in traps regardless of the Hs lure used in this study, suggesting that the species was not present at the time of our experiments.

Role of 14:Ald and Z9-14:Ald Both 14:Ald and Z9-14:Ald are found in the pheromone glands of Hv and Hs, and Z9-14:Ald is the critical secondary sex pheromone component of Hv (Table 2) (Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Klun et al., 1979, 1980a, 1982; Teal et al., 1986; Groot et al., 2005). It is not known, however, whether they are released by calling Hs females, and their contribution to conspecific attraction had not been investigated. Vickers (2002) showed that 0.05–50% Z9-14:Ald (relative to Z11-16:Ald) failed to attract Hs males in a wind tunnel, but in his experimental design Z9-14:Ald replaced the critical secondary pheromone component Z9-16:Ald. Since Hv is abundant in Eastern US and scarce in Western Mexico, we expected blends containing 14:Ald and Z9-14:Ald to be less attractive to Hs males in the former site, if these compounds are used by Hs males to distinguish Hs from Hv blends. However, while their addition did not affect catches in the Eastern United States, they significantly reduced the attractiveness of lures in Western Mexico (Table 6). This suggests that these do not to function as pheromone components for the intraspecific attraction in either region, but it would be instructive to conduct dose-response studies with each of these compounds in HsM to rule out the possibility that they only function optimally at very precise release ratios.

Role of Z11-16:OH In Western Mexico, the response of Hs males to different doses of Z11-16:OH to the lure depended on whether it was added to the HsC or HsM blend (Fig. 2). In the minimal blend, 1% Z11-16:OH caught more Hs males than any other treatment, while in the complete blend a range of doses between 1 and 25% Z11-16:OH caught similar numbers of Hs males. These results suggest that males respond more precisely to component ratios when tested in a minimal blend in the field, whereas in a more complex blend, males respond to broader component ratios as there might be some redundancy of components (Linn and Roelofs, 1989). We, therefore, suggest that the importance of heliothine pheromone compounds may be better discerned when compounds are added singly to a minimal blend in dose–response studies, rather than when compounds are omitted from a complete blend. This approach will be used in future studies to elucidate the optimal percentage of Z11-16:OH needed to attract Hs males in Eastern US, as well as the role of other compounds, such as 16:Ald and Z7-16:Ald, that are present in Hs pheromone glands.

Our results confirm the importance of Z11-16:OH to Hs males, but differ from previously published data. Vickers (2002) found no difference in male responses when Z11-16:OH varied from 1 to 50% in HsM on filter papers in wind tunnel assays, but it is

possible that more replications would be needed to delineate subtle dose-response effects in no-choice wind-tunnel tests. In the field, Heath et al. (1990) reported significantly higher attraction of HsC to rubber septa when Z11-16:OH was loaded between 3 and 6%. The broader range we observed may be related to close-range male orientation. We used Hartstack traps that captured males before they reach the pheromone source, whereas Heath et al. (1990) used sticky and bucket traps, which require that males land close to the lure to be captured. The requirement for close-range orientation to sticky traps may produce sharper dose–response curves, even to the complete blend. In addition, the climatic conditions in Western Mexico at the time that we conducted our experiments may have been different from those when Heath et al. (1990) ran their experiments in Florida, which could differentially affect the release rates of compounds.

Geographic variation in the response of pest moths to sex pheromone blends has important practical implications. Pheromone traps are widely used to monitor pest moth populations with the catch data being used to determine if and when insecticide applications are needed. Geographic differences in male response have led to the development of region-specific pheromone lures for several other moth species (e.g., Klun and Cooperators, 1975; Arn et al., 1983; Hansson et al., 1990; Cork et al., 1992; Tóth et al., 1992; McElfresh and Millar, 1999, 2001; Gemeno et al., 2000; Gries et al., 2001; El-Sayed et al., 2003). Our finding of geographic variation in Hs male response indicates that the development of region-specific lures is an important consideration for heliothine moths as well. Monitoring the presence of Hs with pheromone traps can be a first step towards improved management of Hs populations, especially in tomatillo fields where it is an important pest. Understanding the evolution of pheromone communication in this context is critical because changes in population densities may result in shifts in both the pheromone blend and behavioral responses of males.

Acknowledgments We thank Dr. Ricardo Ayala from the Biological Research Station of the University of Mexico in Chamela, Jalisco, for all logistical help in Jalisco, Pepe Orozco (Aútlan), Vittorio Rincon (Chamela), Francisco Moreno (Hidalgo), Randy Bailey (Oxford), and Erwin Massengil (Smithfield) for granting access to their fields, Reid Evans and Cathy Herring from Central Crops Research Station Clayton, NC, and Nicole Benda and Jen Petzold for assisting with maintenance of the field plot in Clayton, and Scott Bowdridge for logistical help in North Carolina. This research was supported by grants from NSF Population Biology 0235400, the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES), grant # 2005-00896, the W.M. Keck Center for Behavioral Biology, and the Blanton J. Whitmire Endowment at North Carolina State University.

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J Chem Ecol (2007) 33:369–389
DOI 10.1007/s10886-006-9229-2

Role of (3*Z*,6*Z*,8*E*)-Dodecatrien-1-ol in Trail Following, Feeding, and Mating Behavior of *Reticulitermes hesperus*

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Received: 22 November 2005 / Revised: 26 October 2006 / Accepted: 9 November 2006 /
Published online: 3 January 2007
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Abstract Trail pheromones mediate communication among western subterranean termites, *Reticulitermes hesperus* Banks. Repetitive passages of ≥28 termites were required to establish a pheromone trail and trails needed to be reinforced because they lasted <48 hr. The minimal threshold concentration for inducing responses from termite workers and secondary reproductives was between 0.01 and 0.1 fg/cm of (3*Z*,6*Z*,8*E*)-dodecatrien-1-ol (henceforth, dodecatrienol). Workers showed optimal trail-following behavior to dodecatrienol at a concentration of 10 fg/cm. Trails with concentrations >10 pg/cm were repellent to workers. Workers did not detect pheromone gradients, responding equally to increasing or decreasing gradients of dodecatrienol, and termite workers were not able to differentiate between different concentrations of dodecatrienol. Termites preferred dodecatrienol trails to 2-phenoxyethanol trails. Antennae played a key role in trail pheromone perception. Dodecatrienol acted as an arrestant for worker termites (10 fg/cm^2) and male alates (5 ng/cm^2), whereas sternal gland extracts from females attracted male alates. Workers and alates, upon contact with filter paper disks treated with higher doses (10 fg/cm^2 and 5 ng/cm^2, respectively) of dodecatrienol, were highly excited (increased antennation and palpation) and repeatedly returned to the treated disks. Dodecatrienol did not act as a phagostimulant when offered on a paper towel disk. *Reticulitermes hesperus* is highly responsive to dodecatrienol, and it may play an important role in orientation of workers and alates.

Keywords Antennae · Arrestant · (3*Z*,6*Z*,8*E*)-Dodecatrien-1-ol · Phagostimulant · Trail pheromone

Introduction

Semiochemicals play important roles in termite colonies, where they mediate caste regulation, reproduction, nestmate recognition, and behaviors associated with defense and foraging (Prestwich, 1988; Pasteels and Bordereau, 1998). In the subterranean termites *Reticulitermes*

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virginicus Banks, *Reticulitermes lucifugus grassei* Clement, *Reticulitermes speratus* Kolbe, *Reticulitermes santonensis* Feytaud, and *Coptotermes formosanus* Shiraki, the trail pheromone or at least one of its components has been identified as (3*Z*,6*Z*,8*E*)-dodecatrien-1-ol (henceforth, dodecatrienol) (Matsumura et al., 1968, 1969; Honda et al., 1975; Laduguie et al., 1994a; Tokoro et al., 1994; Wobst et al., 1999). In addition, wood degraded by the fungus *Gloeophyllum trabeum* Persoon is reported to be attractive to subterranean termites (Watanabe and Casida, 1963; Matsumura et al., 1969; Rust et al., 1996; Su, 2005), and extracts of the infested wood were found to contain dodecatrienol (Matsumura et al., 1969). Termites other than *Reticulitermes* spp. display trail-following behavior in response to dodecatrienol (Matsumura et al., 1972; Howard et al., 1976; Yamaoka et al., 1987; Tokoro et al., 1989, 1994; Bordereau et al., 1991; Laduguie et al., 1994a, b; Batista-Pereira et al., 2004) and its aromatic analogs (Tai et al., 1971; Prestwich et al., 1984). *Coptotermes formosanus* and *Reticulitermes* spp. also exhibit trail-following behavior toward 2-phenoxyethanol (Chen et al., 1998), a component in ballpoint pen ink. However, the importance of this compound to termites in their natural habitats remains unknown.

After fractionation of extracts of the fourth and fifth sternites of *Reticulitermes hesperus* workers, Grace et al. (1995) demonstrated that a single major component was responsible both for induction of trail following and for orientation of termites on the trail. Studies suggest that other minor components in trail pheromones may lead to qualitative and quantitative differences in the actual trail laid down by worker termites (Howard et al., 1976; Kaib et al., 1982; Runcie, 1987; Tokoro et al., 1994; Cornelius and Bland, 2001; Arab et al., 2004). It has been suggested that time, number of termites laying the trail, and presence or absence of a food source also has an effect on trail quality and longevity (Runcie, 1987; Cornelius and Bland, 2001). Different aspects of termite trail-following behavior have been studied using trails made from natural pheromone (sternal gland extracts [SGE]), synthetic dodecatrienol, and 2-phenoxyethanol (Traniello, 1982; Grace et al., 1988, 1995; Grace, 1991; Chen et al., 1998; Appel and Hu, 2005; Fe et al., 2005). Minor components might also provide the basis for species- and caste-specific responses (Affolter and Leuthold, 2000; Gessner and Leuthold, 2001), and quantitative differences in the concentration of trail pheromone were proposed for differential response in the recruitment behavior of soldiers and worker termites in *Nasutitermes costalis* Holmgren (Traniello and Busher, 1985).

Trail pheromones have been reported to have secondary roles as sex pheromones in various termite species. In *R. santonensis* Feytaud (Laduguie et al., 1994b) and *Cornitermes bequaerti* Emerson (Bordereau et al., 2002), it has been suggested that dodecatrienol is a component of the alate female's sex pheromone. The analogous alcohol, (3*Z*,6*Z*)-dodecadien-1-ol, has been identified as a sex and trail pheromone in *Ancistrotermes pakistanicus* Ahmad (Robert et al., 2004). So far, dodecatrienol has not been shown to be either a trail or sex pheromone of *R. hesperus*, and we did not detect dodecatrienol by GC/mass spectroscopy (MS) analysis of solid phase microextraction extracts of workers and female alates of *R. hesperus* (unpublished data). However, *R. hesperus* has been shown to be very responsive to dodecatrienol, which has been reported to elicit trail-following behavior at a threshold of 6 fg/cm (Matsumura et al., 1972). Grace (1986) was able to isolate trail pheromone from excised sternites by sequential high-performance liquid chromatography fractionation. Based on a GC and subsequent MS analysis of the active fraction and synthetic standards [(6*E, 8Z*- and 6*E,8E*)-dodecadien-1-ol], Grace (1986) concluded that an unsaturated dodecatrienol was present in the active fraction. However, its structure could not be elucidated because of the minute quantities. We also were unable to

detect dodecatrienol in solvent extracts of sternal glands of workers by GC-MS analysis, almost certainly because of the minute quantities of dodecatrienol produced by and present in sternal glands. However, previous studies across several species of subterranean termites indicate that dodecatrienol elicits trail-following behavior and is likely a trail pheromone component. Thus, we tested behavioral responses of workers and males to dodecatrienol with the goal of investigating the role of dodecatrienol as a possible pheromonal signal for *R. hesperus*. Our specific objectives were: (1) to study the preferences and minimal thresholds for trails of natural pheromone (SGE), synthetic trail pheromone (dodecatrienol), and 2-phenoxyethanol; (2) to determine whether termites could detect increasing or decreasing gradients of dodecatrienol; (3) to determine the potential role of dodecatrienol as a feeding stimulant and arrestant; and (4) to assess the responses of alate and dealate adults to synthetic dodecatrienol and to SGE from females.

Methods and Materials

Termites

Western subterranean termites, *R. hesperus* Banks, were collected on the University of California, Riverside Campus in polyvinyl chloride (PVC) traps buried in the ground and provisioned with rolls of corrugated cardboard. Termites were gently removed and maintained in the laboratory in a plastic container (43.3×29.9×17.7 cm, Rubbermaid Inc., Wooster, OH, USA) at 24°C and 98% RH. Pieces of brown paper towel (Fort James Corp., Deerfield, IL, USA) were regularly provided as a food source. Undifferentiated larval termites [mean weight 1.6±0.44 mg (N=50)] were used for bioassays (Thorne, 1996). All worker and soldier termites used in bioassays were from the same colony.

Reticulitermes hesperus alates used in the study were collected from the PVC traps before they swarmed and during swarms. The wings of alates collected from the traps 2–3 d before a swarming event were still wet, grayish-white in color, and the alates were unable to fly. Alates collected from active swarms were kept in the dark inside a plastic petri dish provisioned with a moistened brown paper towel to avoid desiccation. Alates were allowed to acclimate for 1 hr, and individuals that shed their wings (dealates) were used for bioassays.

Termites were starved by confining them inside a plastic petri dish (5.0×0.9 cm, Falcon, BD Biosciences, Franklin Lakes, NJ, USA) containing 5 g of sand moistened with 1 ml of deionized water. One hundred termites were transferred to each of five dishes. Termites were held without food for 24, 48, 72, 96, and 120 hr. Control dishes were provisioned with brown paper towel disks (3.0 cm diam.) as a food source. All the petri dishes were transferred to a plastic container and held at 100% RH. Humidity indicator strips (Sude-Cheme Performance Packaging, Colton, CA, USA) were taped to the walls of the container to ensure that RH remained at 100% throughout the studies. To ensure that termites did not feed on dead individuals, we removed dead or moribund termites regularly during the starvation period.

Sternal Gland Extracts

Fifty worker termites were frozen at −20°C for 5 hr. Sternal glands were dissected from the fourth and fifth abdominal sternites under a binocular stereoscopic microscope (Grace et al., 1988). Dissected glands was transferred to a 2-ml amber glass vial with a Teflon-lined cap

(Wheaton, Millville, NJ, USA) kept at 0°C. The combined glands were extracted overnight in 1 ml *n*-hexane. The supernatant was used for bioassays, with 20 µl considered one termite SGE equivalent. Sternal gland extract from the female alates (N=50) was obtained following the same procedure.

Production of Natural Trails

To determine how many termites were required to establish a pheromone trail, individual termites were placed at one end (release end) of a Y-maze (described under "Choice Bioassays") and were allowed to walk on one arm of the maze. At the intersection, a surgical blade was inserted halfway (0.5 mm) inside one of the two Y-arms to ensure that termites walked only on one arm. To test for trail pheromone, the surgical blade was removed and a termite was placed at one end of the Y-maze and allowed to walk up the trail. Thirty termites were tested, recording the arm chosen by each individual. The test was repeated using trails laid by 5, 10, 15, 20, 25, and 30 termites on the treatment arm. The passage of 30 termites generated a significant response. To narrow the range, we repeated the tests by increasing the number of termites in increments of 1 after 25 termites. The procedure was repeated on the other arm of a new Y-maze to avoid any directional biases.

All Y-mazes treated with different treatments and naturally laid trails were stored separately in sealed plastic containers at room temperature to avoid any direct effects of light on the laid trails. The mazes were tested when the trails were 0.1, 24, 48, and 60 hr old.

Straight-line Bioassays

Straight-line bioassays were conducted to determine the concentration (fg/cm) of dodecatrienol to which termites exhibited optimal trail-following responses. Lines were drawn (27 cm long, <1 mm wide) with a 0.5-mm mechanical pencil on a piece of white printer paper (21.6×27.9 cm, Boise™ X-9™, Boise, ID, USA). The line was lightly marked with a pencil 1 cm from each end of the 27-cm line. This 25-cm-long section was used as the walking distance for each bioassay. These lines were drawn very lightly, and preliminary runs were conducted to ensure that termites did not use them as guidelines. A 10-µl syringe (Hamilton Company, Reno, NV, USA) was used to treat these lines (27 cm) with hexane solutions of test treatments at the desired concentration (wt/cm) of a particular treatment.

For each test run, 100 termites were transferred to a plastic petri dish (3.5×1.0 cm) provisioned with a moistened brown paper towel disk. Termites were allowed to acclimate for ~30 min. A small acrylic paintbrush (no. 3/0 Round Red Sable, M. Grumbacher, NJ, USA) was used to gently put a termite at one end of the treated line. A stopwatch was used to record the time taken by termites to reach the other end of the trail. Timing began as soon as the termite's abdomen crossed the marked starting point of the 25-cm treated section of the trail, and ended as soon as it crossed the opposite end point. The number of termites that did not walk to the end of the trail, left the trail, or reversed their direction was recorded. Time recordings of these termites were not included in the rate of movement analysis.

Seven concentrations of dodecatrienol (0.01 fg/cm, 0.1 fg/cm, 1 fg/cm, 10 fg/cm, 100 fg/cm, 1 pg/cm, and 10 pg/cm) were tested with 30 termites per concentration. To ensure that termites did not detect any natural pheromone from a previous run, a new sheet of paper and a dodecatrienol trail were used for every run.

Gradient Bioassays

To determine whether termites responded to increasing or decreasing dodecatrienol concentration gradients, trails were drawn on printer paper as described above. The 25-cm long section was further divided into five equal sections. We selected a range of concentrations between 1 and 10 fg/cm to construct the gradient trail. Each 5-cm section was treated with an increasing concentration of dodecatrienol (1, 2, 5, 8, and 10 fg/cm). A single termite was placed at either the low concentration (increasing gradient) or the high concentration end (decreasing gradient). The termite's walking speed, pauses, and reversals in direction and the number of termites leaving the trail were observed. A new gradient trail was constructed on a new sheet of paper for each run. Thirty termites were tested on increasing and decreasing gradient trails.

Choice Bioassays

To study different aspects of trail-following behavior of termites, natural trails (laid down by worker termites), SGE trails, and artificial trails with dodecatrienol were used. A Y-maze was designed from a cardboard sheet (1 mm thick), with each arm of the Y-maze being 120° apart, 5 cm in length, and 3 mm in width (Fig. 1). All the Y-mazes were cut on a die-cutting machine such that each maze had a well-perforated margin. A no. 7 insect mounting pin (Ward's Natural Science, Rochester, NY, USA) was gently inserted in the Y-maze at the center at the junction of the three arms. The other end of the pin was inserted into a Styrofoam block. The Styrofoam served as a base to hold the Y-maze stable so that the arms were level. A 10-µl syringe was used to treat the surface of one arm with solutions of the desired concentration (wt/cm) of a particular treatment. The other arm of the maze was treated with acetone or hexane only. Three concentrations of SGE (TE = termite equivalent) (0.002, 0.02, and 0.2 TE/cm) and seven concentrations of dodecatrienol (0.01 fg/cm, 0.1 fg/cm, 1 fg/cm, 1 pg/cm, 0.01 ng/cm, 0.1 ng/cm, and 1 ng/cm) were tested. In other studies, different concentrations of dodecatrienol were applied to the two arms of the Y-maze to determine termites' ability to discriminate between different concentrations. Similarly, 2-phenoxyethanol

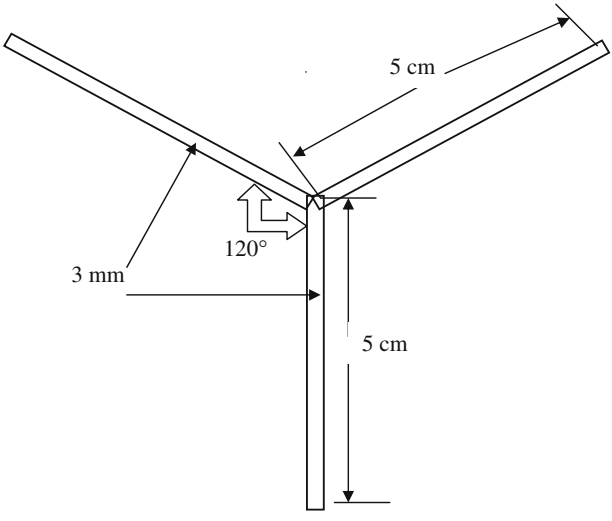


Fig. 1 Diagram of the Y-maze used for choice bioassays. Each arm is 120° apart, 5 cm long, and 3 mm wide

[ethylene glycol phenyl ether, (90+ percent purity), Aldrich Chemical Company, Inc., Milwaukee, WI, USA] trails were laid down using three concentrations (0.02 ng/cm, 0.2 ng/cm, and 10 μg/cm) on one arm and compared with the untreated arm as well as with different dodecatrienol concentrations.

The same acrylic paintbrush described above was used to gently put a termite at the end of one of the untreated arms, and the worker walked up to the intersection and chose between the treated or untreated arm. The run was considered complete when the termite walked to the end of one 5-cm arm. A new termite was used for each run, and 30 runs were made for each test. To control for directional biases, 15 runs were conducted with the left arm treated and 15 runs with the right arm treated. A new Y-maze was used for each run. All bioassays were performed in a room (6.70×3.65×3.35 m) illuminated by four 32-W white fluorescent tubes. We conducted preliminary runs in the absence of light and determined that light did not influence the trail following ability of *R. hesperus* workers (without functional eyes) and male and female dealates.

Detection of Trail Pheromone

To determine how termites detect trail pheromone, termites were tested on a bilevel Y-maze arena. A Y-maze with one arm treated as described above was inverted and rested upon three insect-mounting pins inserted in a Styrofoam sheet so that the Y-maze was level (Fig. 2). The arms of another Y-maze were cut such that each arm measured 4 cm. A no. 7 pin (cut to 3.7 cm in length) was inserted through a cork such that the pointed end was about 2 cm out on the other side. The pin and cork assembly was carefully pressed onto the center of the shorter Y-maze so that it securely held the shorter Y-maze. The shorter Y-maze was put directly under the inverted Y-maze (one arm treated) described above (Fig. 2). The distance between the untreated surface of the arms of the shorter Y-maze and the inverted Y-maze above it was ~1.0 cm. A single termite was placed on the end of the bottom Y-maze. The termite's behaviors were observed when it came to the intersection, specifically looking for any antennal response toward the treated arm above its head. The run was terminated when a termite walked to the end of the 4-cm arm of the shorter Y-maze (Fig. 2). For the series of assays, the distance (4 to 10 mm) between the treated inverted Y-maze and untreated smaller Y-maze surfaces was adjusted by inserting 1-mm-thick disks (2.5 cm diam.) of cardboard under the cork holding the smaller Y-maze.

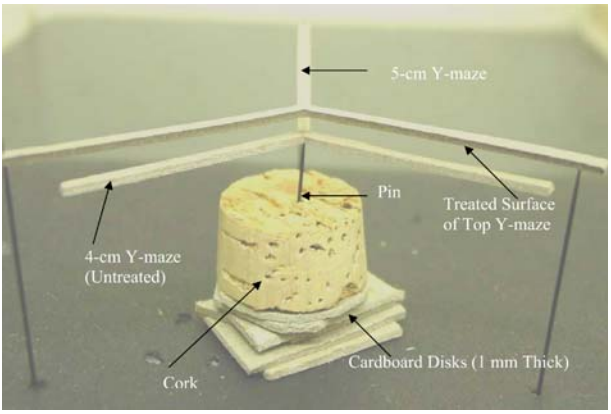


Fig. 2 A bilevel Y-maze used to determine whether the antennae or palpi detected the trail pheromone. One arm of the top Y-maze (each arm 5 cm long) was treated with acetone solutions of dodecatrienol and the other with acetone only. The arms of the shorter Y-maze were not treated. The distance between the top and bottom Y-mazes was adjusted by putting 1-mm-thick cardboard disks under the wooden cork

Arrestant Bioassays

To determine whether dodecatrienol or SGE arrested termite movement, four separate bioassays were conducted with workers and male dealates. The bottom of a plastic petri dish (9.0 cm diam.) was completely covered with a filter paper disk (Whatman no. 1, Whatman International Ltd., Maidstone, UK). For the first bioassay, a small filter paper disk (2.1 cm diam., Whatman no. 1) was treated with 10 μl of a solution of dodecatrienol in acetone (3.46 pg/ml) using a 10-μl syringe (Hamilton). The resulting concentration of dodecatrienol on the treated disk was 10 fg/cm^2. Another filter paper disk (2.1 cm diam.) was treated with 10 μl of acetone only. Both disks were allowed to dry for 5 min under a laminar flow hood and then glued to the bottom of the larger disk (9.0 cm diam.) so that they were 5 cm apart (between their center points). Individual workers were released in the center of the arena. The amount of time each termite (N=15) spent contacting each disk was recorded for 5 min. Their gnawing and palpation behaviors on the treated disk were also observed. New disks were used for each run.

In the second bioassay, the attractiveness of dodecatrienol to male dealates (N=15) was tested. Preliminary bioassays at different concentrations ranging from 10 fg/cm^2 to 10 ng/cm^2 were tested, and 5 ng/cm^2 was selected for a full experiment. The amount of time spent by male dealates contacting each disk was recorded for 5 min. Their palpation, excitement, and gnawing behaviors on treated vs. untreated disks were visually observed. The third bioassay was carried out using SGE from female dealates (1 female alate equivalent) on one disk and hexane (control) on the other disk. Male dealates (N=15) were released in the arena one at a time as described above. The amount of time each male spent contacting each disk was recorded for 5 min. Their palpation, excitement, and gnawing behaviors on treated vs. untreated discs were visually observed. The fourth bioassay was conducted to compare attractiveness of dodecatrienol with that of female SGE, for male dealates (N=15). The amount of time each male spent contacting each disk was recorded for 5 min. Their palpation, excitement, and gnawing behaviors on treated vs. untreated discs were visually observed. Termite workers used in the first bioassay were taken from the lab colony in cohorts of 50 termites, and were transferred to a plastic petri dish (3.5 cm diam.). The disk was provisioned with a moistened brown paper towel disk. Termites were allowed to acclimate for 30 min. Termites were used only once. The alates used were collected from an active swarm. They were immediately transferred to a plastic container provisioned with moistened brown paper towel, and dealates separated by sex were used for bioassays.

Feeding Bioassays

To determine whether dodecatrienol acted as a phagostimulant, workers were tested in a feeding choice bioassay. Small disks of brown paper towel (2.5 cm diam.) were cut in half, and both halves were weighed. One half was designated as treated (T) and the other as control (C). A calculated volume of dodecatrienol solution in acetone was applied to the treated half disk with an Eppendorf micropipetter to provide the desired concentration (pg/cm^2). The untreated half was treated with the same volume of acetone. Both disk halves were dried under a laminar flow hood for 5 min and were then weighed again. Concentrations of 0.01, 0.1, 1, and 10 pg/cm^2 and 1 ng/cm^2 were tested. After drying, treated and untreated half disks were stuck back together with a small strip of Scotch tape (3M®, St. Paul, MN, USA). The intact disk was placed in a plastic petri dish (3.5 cm diam.×1 cm, Falcon, BD Biosciences) with the taped side facing down. All the petri dishes with disks were then transferred to a plastic container (43.3×29.9×17.7 cm) and were maintained at 100% RH.

Forty termites of similar size and weight were transferred to each petri dish, the dishes were covered with lids to keep moisture high, and the dishes were held in the container for 5 d. Appropriate controls were used to check for mortality and amount of feeding on disks treated with acetone only. Five replicates were conducted for each concentration. At day 5, the termites were removed, and the two halves of the disk were separated, cleaned by gently brushing the fecal material and dirt from them, and dried in a laminar flow hood for 24 hr. The half disks were weighed again. Net feeding was calculated after subtracting amount of feeding on control halves from the feeding on treated halves.

Statistical Analyses

Statistical analyses of choice tests on Y-mazes were based on a binomial distribution, using a chi-square test ($P<0.05$) (SAS Institute, 1999). Results of termite feeding and straight-line bioassays were analyzed with the general linear model analysis of variance (ANOVA) procedure (SAS Institute, 1999). Means were separated using Tukey's honestly significant difference (HSD) test for significance at $P<0.05$. Arrestant bioassay data were analyzed by paired *t*-tests.

Synthesis of (3*Z*,6*Z*,8*E*)-Dodecatrien-1-ol (Fig. 3)

Unless otherwise stated, reactions were run under argon atmosphere. Tetrahydrofuran (THF) was dried by distillation from sodium-benzophenone ketyl. Solutions were dried over anhydrous Na_2SO_4 and concentrated with rotary evaporation under reduced pressure, unless otherwise stated. NMR spectra were taken on a Varian INOVA 400 NMR spectrometer, and mass spectra were taken with a Hewlett-Packard 6890 gas chromatograph interfaced to a Hewlett-Packard 5973 mass selective detector, using electron impact ionization at 70 eV. Reaction yields have not been optimized.

*(E)-1-Iodo-1-pentene (**3**)* Diisobutylaluminum hydride in hexanes (1 M, 100 ml, 100 mmol) was added dropwise to a solution of 1-pentyne **2** (7.5 g, 110 mmol) in hexanes chilled to −40°C. The resulting mixture was stirred 30 min, then slowly warmed to room temp over 3 hr, and stirred overnight. The mixture was warmed to ~50°C for 4 hr, then cooled to

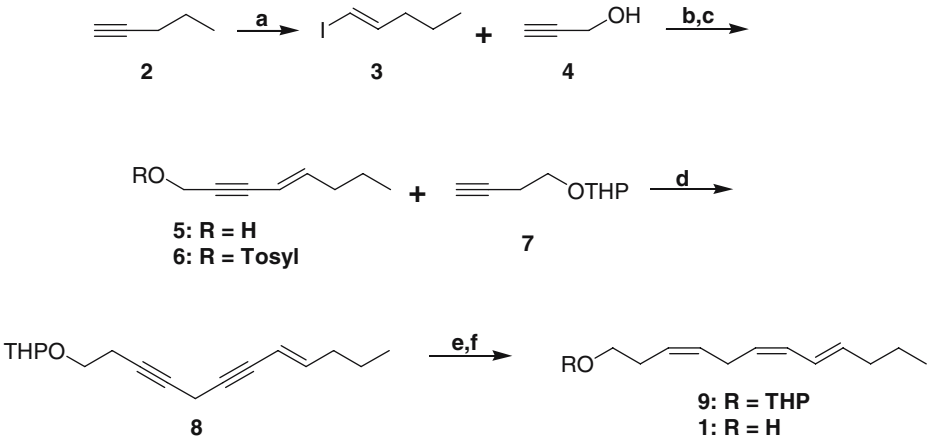


Fig. 3 Synthesis of dodecatrienol

−40°C, and a solution of iodine (25.4 g, 100 mmol) in THF (100 ml) was added dropwise over 30 min. The resulting dark brown suspension was allowed to warm to room temperature with stirring overnight, producing a clear pale yellow solution. The solution was cooled in an ice bath, and slowly quenched by dropwise addition of ice-cold H_2SO_4 (20%, 50 ml) (CAUTION: EXOTHERMIC) with vigorous stirring. After stirring for 30 min, the layers were separated, and the organic layer was washed with dilute $NaHSO_3$ solution and brine, and dried over $MgSO_4$. The resulting pale yellow solution was filtered through a 2.5-cm pad of silica, rinsing with pentane, then concentrated by rotary evaporation without heating. The resulting pale orange oil **3** (28.9 g, 88.8% pure) was contaminated with isobutyl iodide (8.7%), 1-iodo-1-pentyne (0.8%), and iodopentane (1.8%), but appeared to be isomerically pure. The crude material was used without further purification. MS: *m/z* (%): 196 (M+, 40), 167 (22), 154 (28), 127 (13), 69 (20), and 41 (100).

*(E)-Oct-4-en-2-yn-1-ol (**4**)* A 250-ml three-neck flask fitted with a thermometer was flushed with Ar and loaded with bis(triphenylphosphine)palladium (II) dichloride (1 g, 1.4 mmol), 0.5 g CuI (2.6 mmol), and pyrrolidine (50 ml). The mixture was stirred and cooled in an ice bath, and crude (*E*)-1-iodo-1-pentene **3** (14.5 g, ~50 mmol) was added dropwise, followed by dropwise addition of propargyl alcohol **4** (3.5 ml, 60 mmol). The mixture was slowly warmed to room temperature, at which point a rapid exothermic reaction commenced, necessitating external cooling with an ice bath. After cooling the mixture to room temperature, checking the mixture revealed a clean reaction with complete consumption of iodoalkene **3** and production of a single product. The reaction mixture was worked up by pouring into an ice-cold 1:1 mixture of saturated aqueous NH_4Cl and 2 M HCl (300 ml), and extracting several times with ether. The combined ether extracts were washed with a small portion of the NH_4Cl and HCl mixture and brine, dried and decolorized with a mixture of anhydrous Na_2SO_4 and decolorizing charcoal, filtered through a plug of celite, and concentrated. The resulting viscous orange oil was Kugelrohr-distilled (oven temp ~70°C, 1 mmHg), yielding 3.5 g of clear oil (56%). MS: 124 (M+, 96), 109 (15), 95 (42), 91 (40), 81 (100), 79 (40), 77 (34), 67 (40), 65 (41), 55 (28), 54 (26), 53 (31), 41 (48). ^{1}H NMR: 6.14 (dt, 1H, J=15.97, 7.04 Hz, H-5), 5.48 (dtt, 1H, J=15.97, 2.05, 1.5 Hz, H-4), 4.35 (d, 2H, J=2.05 Hz, H-1), 2.09 (overlapped tdd, 2H, J~7.5, 7.1, 1.5 Hz, H-6), 1.91 (br s, 1H, OH), 1.41 (overlapped qt, 2H, J~7.5, 7.3 Hz, H-7), and 0.89 (t, 3H, J =7.3 Hz, H-8). ^{13}C NMR: 145.8, 109.1, 85.8, 84.8, 51.8, 35.3, 22.1, and 13.8.

*(E)-Oct-4-en-2-yn-1-ol Tosylate (**6**)* Enynol **5** (3.1 g, 25 mmol) and *p*-toluenesulfonyl chloride (5.4 g, 28 mmol) were dissolved in 100 ml of ether, and the solution was cooled to ~−10°C in an ice–salt bath. With vigorous stirring, powdered KOH (16.8 g, 300 mmol) was added in portions over 30 min, and the resulting mixture was warmed to ~ 0°C and stirred until the starting alcohol had been completely consumed (~1 hr). The mixture was then poured into 100 ml of ice water, stirred for 10 min, and the layers were separated. The aqueous layer was extracted again with ether, and the combined ether extracts were washed with brine, dried, concentrated, and then pumped under high vacuum (~0.2 mmHg) for 1 hr to remove traces of solvent, yielding the tosylate as a gold oil (6.32 g). This was used immediately in the next step without further purification.

Isopropyl magnesium chloride (2 M, 16 ml, 32 mmol) was added dropwise at room temperature to a solution of THP-protected 3-butyn-1-ol **7** (4.62 g, 30 mmol) in 75 ml THF. The mixture was stirred for 2 hr at room temperature, then cooled to 0°C, and CuI (0.5 g,

2.6 mmol) was added. After stirring for 15 min, crude tosylate **6** (3.2 g, ~12.5 mmol) in 20 ml of THF was added dropwise. The mixture was warmed slowly to room temperature overnight in an insulated bath, then worked up by addition of saturated NH_4Cl and extraction with hexane. The hexane extract was washed with brine and dried over Na_2SO_4, filtered, concentrated, and Kugelrohr-distilled (oven temperature <90°C, 0.2 mmHg) to remove the excess THP-protected butynol. The residue was then purified by vacuum flash chromatography (7.5% ethyl acetate [EtOAc] in hexanes), yielding the protected endiynol **8** (1.06 g), which was used immediately in the next step.

A solution of dicyclohexylborane was prepared by addition of cyclohexene (2.43 ml, 24 mmol) dropwise to a THF solution (20 ml) of borane-dimethylsulfide complex (2 M, 6 ml, 12 mmol) maintained at <−10°C in an ice–salt bath. After the addition was complete, the mixture was slowly warmed to room temperature over 3 hr, yielding a white slurry. The mixture was cooled to <−10°C again, and the protected enediynol (0.78 g, 3 mmol) in 10 ml THF was added dropwise. The solution was slowly warmed to 0°C over 3 hr, and stirred at 0°C for a further 3 hr, yielding a cloudy orange solution. Acetic acid (3 ml, 50 mmol) was then added dropwise, and the solution was warmed to room temperature overnight. The next morning, the mixture was cooled to 0°C again, and a solution of 4 g NaOH in 20 ml H_2O was added over 10 min, followed by dropwise addition of 1.4 ml of 30% H_2O_2 (EXOTHERMIC). The resulting mixture was warmed to room temperature and stirred for 30 min, poured into water, and extracted with hexane. After washing with brine, drying over Na_2SO_4, and concentrating, the residue was purified by vacuum flash chromatography (5% EtOAc in hexane). The resulting yellow oil **9** was dissolved in MeOH (15 ml) containing ~50 mg *p*-toluenesulfonic acid, and stirred at room temperature for 3 hr. The mixture was then poured into aqueous $NaHCO_3$ and extracted twice with hexane. The combined hexane extracts were washed with saturated $NaHCO_3$ and brine, then dried over a mixture of anhydrous Na_2CO_3 and Na_2SO_4. After filtration and concentration, the residue was purified by flash chromatography (22% EtOAc in hexane). The purified trienol **1** was Kugelrohr-distilled (oven temperature ~80–90°C, 0.2 mmHg), yielding 280 mg of trienol **1**, >99.5% pure by GC. MS: 180 (29), 137 (8), 133 (10), 119 (25), 105 (48), 93 (41), 91 (100), 79 (73), 77 (37), 67 (40), 55 (28), and 41 (30). ^{1}H NMR: 6.31 (ddq, 1H, J=15.1, 11.0, 1.3 Hz, H-8), 5.97 (br t, 1 H, J=11.1 Hz, H-7), 5.69 (dt, 1H, J=15.1, 7.0 Hz, H-9), 5.56 (ddt, 1H, J=10.7, 7.3, 1.5 Hz, H-4), 5.43 (ddt, 1H, J=10.7, 7.3, 1.5 Hz, H-3), 5.25 (dd, 1H, J=10.8, 7.5 Hz, H-6), 3.66 (br q, 2H, J=6.3 Hz, H-1), 2.95 (br t, 2H, J=7.3 Hz, H-5), 2.37 (br q, 2H, J=6.8 Hz, H-2), 2.09 (br q, 2H, J~7.1 Hz, H-10), 1.42 (overlapped tq, 2H, J~7.3, 7 Hz, H-11), and 0.91 (t, 3H, J=7.3 Hz, H-12). All spectra were in accord with literature values (Eya et al., 1990; Argenti et al., 1994).

Results

Termites (N=30) walked on the Y-maze at an average speed of 0.73±0.12 cm/sec on the untreated arm of the Y-maze where they were released. Once the termites reached the intersection of the maze and turned toward the dodecatrienol treated arm (1 fg/cm), they traveled significantly faster (t=8.08, df=29, P<0.05) with fewer delays toward the end than on nontreated controls. The average speed on the treated arm was 1.05±0.18 cm/sec (N=30).

Production of Natural Trails

Individual termites laid trails that could be detected by other termites in significant numbers only after about 28 termites walked along the given Y-maze treatment arm compared to the

other control arm (χ^2=13.34, *df*=1, *P*<0.001). This trail lasted for 1 d when the maze was held inside a sealed container at room temperature (Table 1). By day 2, the trail no longer elicited a significant response.

Straight-line Bioassays

Termites showed optimal responses to trails of 0.1 to 10 fg/cm dodecatrienol in our bioassays. When exposed to trails made with the various concentrations of dodecatrienol, they did not show significant differences in their mean walking speed on the trail except for the 10 fg/cm trail (*F*=6.91, *df*=5, 29, *P*<0.001; Table 2), on which they walked more quickly (1.15 cm/sec) than they did with most of the other concentrations. The percentage of termites that walked the entire distance of the treatment trails was lower at higher concentrations of dodecatrienol (*F*=17.91, *df*=5, 29, *P*<0.001; Table 2) compared with lower concentrations (≤10 fg/cm).

Gradient Bioassays

There was no difference in trail-following behavior of the termites when they were exposed to increasing or decreasing gradients of dodecatrienol. Their speed walking up or down gradients was not significantly different, with termites covering the distance in about 24 sec (increasing gradient 24.7±5.0 sec and decreasing gradient 23.7±4.5 sec). Similarly, no differences were observed in the percentages of termites (88 to 90%) that completed the gradient trails compared to nongradient trails.

Choice Bioassays

A minimum of 0.02 TE/cm (worker SGE) was required for trail following (Table 3). The threshold concentration of 2-phenoxyethanol to elicit significant levels of trail following on a Y-maze was 0.2 ng/cm. For dodecatrienol the threshold concentration was between 0.01

Table 1 The number of *R. hesperus* walking on the Y-maze arm before other termites followed the trail

No. of termites[a]	Control/Treated	Chi-square[b]
5	18/12	1.2
10	16/14	0.13
15	13/17	0.54
20	14/16	0.13
25	19/11	2.13
26	10/20	3.34
27	10/20	3.34
28	5/25	13.3
29	3/27	19.2
30	6/24	10.8
28 (24 hr)	9/21	4.8
28 (48 hr)	13/17	0.54

[a] Deposits fresh unless indicated by the number of hours later that the bioassay maze was tested after deposition.

[b] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between treated (traveled by termites) and control arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

Table 2 *Reticulitermes hesperus* worker responses to different concentrations of dodecatrienol on a 25-cm-long trail

Concentration	Percent of Termites Completing the Trail	Time (sec) Mean±SD
0.01 fg/cm	0.0a	0.0
0.1 fg/cm	75.0b	28.7±6.5a
1 fg/cm	90.9b	26.0±6.3ab
10 fg/cm	93.0b	21.7±3.8b
100 fg/cm	51.7c	29.5±6.2a
1 pg/cm	48.4cd	26.3±5.8a
10 pg/cm	38.5d	27.4±5.4a

Mean values with the same letter in a column are not significantly different (Tukey's HSD test, $P<0.05$).

and 0.1 fg/cm. Thus, *R. hesperus* workers were 10^7 times more responsive to dodecatrienol than they were to 2-phenoxyethanol (Table 3).

Sternal gland extracts at 0.2 TE/cm elicited significant trail following for only 24 hr after application (Table 3), whereas a 2-phenoxyethanol trail at 0.2 ng/cm on a Y-maze lasted for 24 hr, and a 1 fg/cm trail of dodecatrienol was active for at least 48 hr.

Threshold concentrations for trail following on dodecatrienol trails were similar for workers and secondary reproductives (Table 4). Soldiers and alate males of *R. hesperus* were not able to follow trails at 0.1 fg/cm dodecatrienol (Table 4). Both workers and soldiers followed the 2-phenoxyethanol trails at equivalent threshold levels (0.2 ng/cm). Workers,

Table 3 Responses of *R. hesperus* workers to trails of 2-phenoxyethanol, SGE, and synthetic dodecatrienol on a Y-maze

Compound	Concentration	Age (hr)	Control/Treated	Chi-square[a]
SGE	0.002 TE/cm	0.1	13/17	0.53
	0.02 TE/cm	0.1	3/27	19.2
	0.2 TE/cm	0.1	5/25	13.34
		24	7/23	8.54
		48	14/16	0.13
2-Phenoxyethanol	0.02 ng/cm	0.1	17/13	0.53
	0.2 ng/cm	0.1	4/26	16.13
	0.2 ng/cm	24	7/23	8.54
	0.2 ng/cm	48	10/20	3.34
	10 μg/cm	0.1	6/24	10.8
Dodecatrienol	0.01 fg/cm	0.1	14/16	0.13
	0.1 fg/cm	0.1	8/22	6.54
	1 fg/cm	0.1	1/29	26.1
	1 fg/cm	24	5/25	13.3
	1 fg/cm	48	4/26	16.13
	1 fg/cm	60	20/10	3.34
	1 pg/cm	0.1	6/24	10.8
	0.01 ng/cm	0.1	23/7	8.54
	0.1 ng/cm	0.1	22/8	6.54
	1.0 ng/cm	0.1	24/6	10.8

TE = termite equivalent.

[a] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between treated and control arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

Table 4 Responses of different castes of *R. hesperus* to SGE, 2-phenoxyethanol, and dodecatrienol on a Y-maze

Treatment	Caste	Control/Treatment	Chi-square[a]
SGE (Worker)	Worker	5/25	13.34
0.2 TE/cm	Soldier	3/27	19.2
	SR	4/26	16.13
0.02 TE/cm	Worker	7/23	8.54
	Soldier	9/21	4.8
	SR	7/23	8.54
0.002 TE/cm	Worker	13/17	0.53
	Soldier	16/14	0.13
	SR	14/16	0.13
SGE alate (F) (0.2 TE/cm)	Alate (M)	3/27	19.2
SGE alate (F) (0.02 TE/cm)	Alate (M)	17/13	0.53
2-Phenoxyethanol (0.2 ng/cm)	Worker	4/26	16.13
	Soldier	5/25	13.34
2-Phenoxyethanol (0.02 ng/cm)	Worker	16/14	0.13
	Soldier	13/17	0.53
Dodecatrienol	Worker	1/29	26.13
1 fg/cm	Soldier	3/27	19.2
	SR	2/28	22.53
	Alate (M)	5/25	13.34
0.1 fg/cm	Soldier	10/20	3.34
	SR	9/21	4.8
	Alate	12/18	1.2
0.01 fg/cm	Soldier	13/17	0.53
	SR	16/14	0.13
	Alate	12/18	1.2

SGE = sternal gland extract, TE = termite equivalent, SGE alate (F) = sternal gland extract of female alates, SR = secondary reproductive

[a] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between the control and treated arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

secondary reproductives, and soldiers readily followed the trails of 0.02 TE of SGE/cm of worker termites. Male alates followed the trails of 0.2 female alate SGE/cm (Table 4).

Starvation appeared to have no effect on the sensitivity of termites to dodecatrienol. Those starved for 120 hr were still capable of following 0.1 fg/cm dodecatrienol trails on the Y-maze (χ^2=6.54, *df*=1, *P*<0.013; Table 5). However, those starved up to 120 hr were not able to differentiate between 1 fg/cm vs. 10^4 fg/cm dodecatrienol trails (χ^2=2.13, *df*=1, *P*<0.18; Table 5).

Termites were not able to differentiate between different concentrations of fresh trails of dodecatrienol or SGE when offered simultaneously on each of the two arms of the Y-maze (Table 6). Even when the differences in concentrations of dodecatrienol were as great as 10^4, they did not show any preference. However, significantly more followed the higher concentration trail after it aged for 48 hr.

When given a choice between 2-phenoxyethanol and dodecatrienol, workers generally preferred the latter (Table 7). Only when the concentration of 2-phenoxyethanol was 10^{11} (0.1 mg/cm) or 10^{12} (1 mg/cm) times greater than dodecatrienol (1 fg/cm) did they show significantly higher preference for the 2-phenoxyethanol trails (Table 7). Unlike

Table 5 Trail-following behavior of *R. hesperus* workers starved for different durations

Starvation Period (hr)	Conc. (fg/cm)	Control/Treatment	Chi-square[a]
24	1.0	5/25	13.34
	0.1	7/23	8.54
	0.01	16/14	0.13
24	1.0 vs. 10^4	12/18	1.2
120	1.0	4/26	16.13
	0.1	8/22	6.54
	0.01	14/16	0.13
120	1.0 vs. 10^4	11/19	2.13

[a] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between treated and control arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

dodecatrienol (0.01 to 1 ng/cm, Table 3), at a higher concentration 2-phenoxyethanol (1 mg/cm, Table 7) was not repellent to termite workers.

Detection of Trail Pheromone

In this experiment, a treated Y-maze was placed directly above the untreated Y-maze upon which the test animal walked. Neither the antennae nor the mouthparts of the termites could touch the treated surface of the Y-maze above them. When the distance between the treated and untreated surfaces of the two Y-mazes was reduced to 4 mm, termites were able to detect and follow the treated arm (Table 7). When the distance between the two mazes was reduced to less than 4 mm, they were able to touch the upper Y-maze surface and in few cases were able to climb up the maze over their heads. Dodecatrienol has relatively low vapor pressure (estimated as 3.73×10^{-4} Torr at 25°C; Chemical Abstracts, 2005), and termites were only able to detect it when their antennae were about 1–2 mm from the trail. Increasing the concentration of dodecatrienol did not alter the distance over which it was perceived by the antennae (Table 8). Concentrations as high as 1 ng/cm were not repellent to workers in this setup.

Table 6 Ability of *R. hesperus* workers to differentiate between different concentrations of synthetic dodecatrienol and SGE on the Y-maze

Concentration/cm[a] of Arm A vs. Arm B	Time (hr)	A/B	Chi-square[b]
1 fg/cm vs. SGE 0.2 TE/cm	0.2	13/17	0.53
	24	14/16	0.13
	48	12/18	1.2
1 vs. 100 fg/cm	0.2	16/14	0.13
	24	11/19	2.13
	48	8/22	6.53
1 vs. 10^4 fg/cm	0.2	13/17	0.53
	24	12/18	1.2
	48	9/21	4.8

[a] TE = termite equivalent, Arm A treated with 1 fg/cm dodecatrienol

[b] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between two treated arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

Table 7 Ability of *R. hesperus* workers to differentiate between synthetic dodecatrienol and 2-phenoxyethanol trails

Concentration (fg/cm)			Chi-square[a]
Dodecatrienol	2-Phenoxyethanol	Dodecatrienol/2-P	
1	1	28/2	22.53
1	10^3	24/6	10.8
1	10^5	27/3	19.2
1	10^7	24/6	10.8
1	10^9	23/7	8.54
1	10^{10}	16/14	0.13
1	10^{11}	6/24	10.8
1	10^{12}	8/22	6.54

2-P = 2-phenoxyethanol.

[a] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between two treated arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

Arrestant Bioassays

Both worker termites and male dealates spent more time on disks treated with 10 fg/cm^2 and 5 ng/cm^2 dodecatrienol, respectively, than on control disks (Fig. 4A, t=4.45, df=14, P <0.05; Fig. 4B, t=3.61, df=14, P<0.05). Male dealates also spent more time (t=10.62, df= 14, P<0.05) on disks treated with female alate extract (SGE, 0.28 TE/cm^2) than on control disks (Fig. 4C). When female alate SGE was directly compared to dodecatrienol, male dealates spent more time (t=6.10, df=14, P<0.05) on SGE-treated disks (0.28 termite equivalent/cm^2) than disks treated with dodecatrienol (5 ng/cm^2, Fig. 4D).

Feeding Bioassays

The total amount of feeding on treated and untreated paper combined at various dodecatrienol concentrations was not significantly different (F=2.45, df=5, 24, P=0.06). Total feeding on the treated and control halves combined ranged from 5.9 to 3.9 mg 40 termites^{-1} 5 d^{-1}. Net feeding on the treatments was determined by subtracting the amount of feeding on the control half of the disk from feeding on the treated half. Net feeding on treated halves ranged from −1.6 to +1.9 mg 40 termites^{-1} 5 d^{-1}. No consistent trend of feeding on either the control or treated half was observed. There were no significant differences in the amount of net feeding on paper towel disk halves treated with different concentrations of dodecatrienol (F=1.92, df=5, 20, P=0.15).

Table 8 Detection of trail pheromone by *R. hesperus* workers on the double Y-maze arms

Weight/cm on Y-maze Arm	Distance Between Arms (mm)	Control/Treatment	Chi-square[a]
1 fg	10	12/18	1.2
1 fg	5	11/19	2.13
1 fg	4	6/24	10.8
1 ng	5	10/20	3.34
1 ng	4	7/23	8.54

[a] Chi-square (χ^2) analysis is based on a binomial probability of 1:1 for a null hypothesis of no difference between two treated arms, and χ^2 values less than 3.84 are not significant at the 0.05 level of significance.

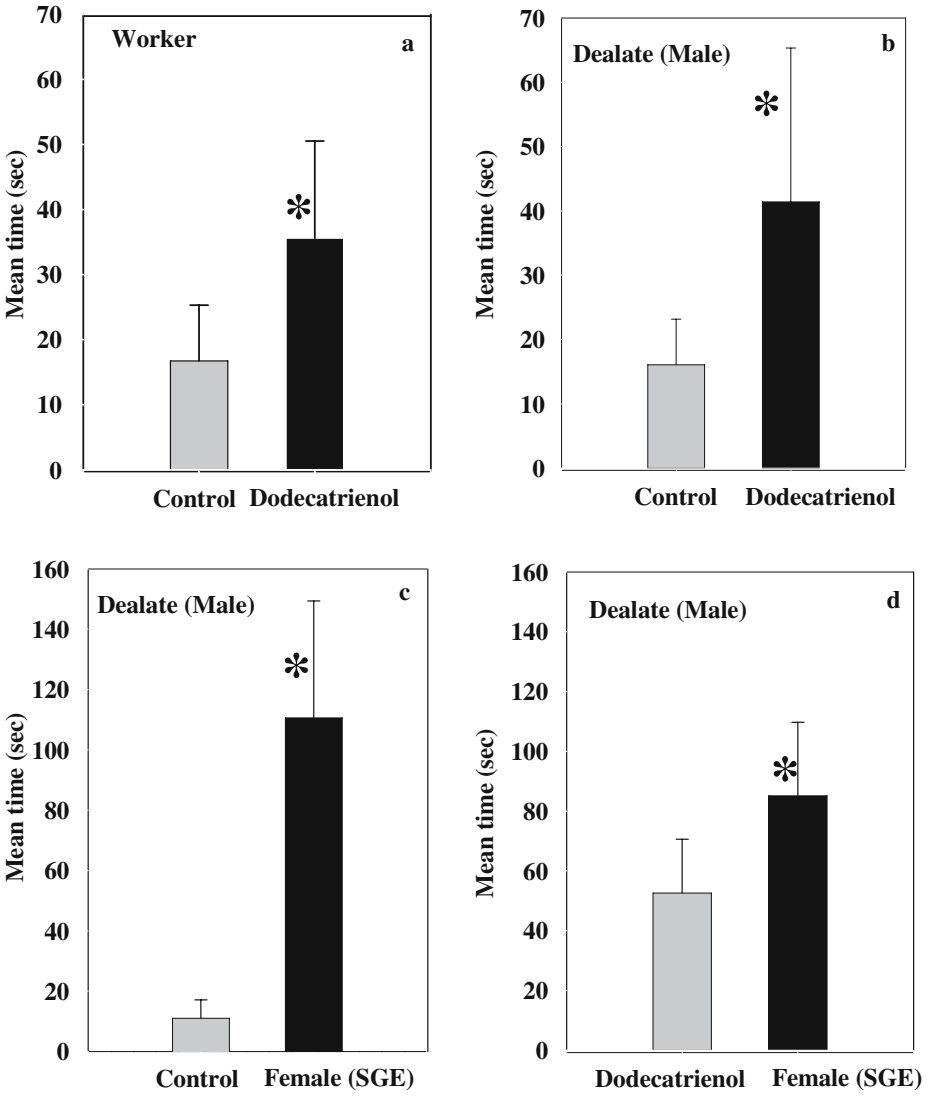


Fig. 4 Time spent (sec) on disks treated with dodecatrienol or female alate SGE by workers (**a**) and male dealates (**b**–**d**). Asterisk denotes means that are significantly different at $P<0.05$ (paired *t*-test)

Discussion

Individual termites, when allowed to walk on the Y-maze substrate, did not establish a trail that was recognized by conspecifics. Instead, it took a minimum of 28 termites walking over the same trail to establish a reliably detectable pheromone trail. This suggests that during its routine movements, a new recruit worker will prefer trails that have had considerable traffic, and these trails must be reinforced to remain effective. Grace et al. (1988) also suggested that *R. hesperus* termites are preferentially recruited to more well traveled trails.

Similar observations were made by Runcie (1987) concerning the trail-following behavior of *R. flavipes*, where trails in tubes with heavier termite traffic lasted longer than trails in tubes that were used less frequently. Single termite workers were allowed to lay trails in glass tubes and superimposed trails of as many as 50 workers remained active for only 5 min, whereas in our study trails superimposed by 28 termites remained active for 24 hr. These differences may be attributed to the properties of the substrate over which the trail was laid. In our study, the Y-maze was made of cardboard instead of a glass surface.

The concentrations of the trails did not affect the speed at which termites completed the trails. However, the concentration did affect the percentage that completed the trail bioassay. It took more than 30 termites to achieve 30 replicates in which the termite walked the entire length of the 25 cm trail because some veered off the trail before reaching the end. Termites exposed to 0.01 and 0.1 fg/cm trails walked slowly and stopped frequently, trying to sense the trail by actively waving their antennae (sampling substrate) on both sides of the trail. There was a limited range of dodecatrienol concentrations (0.1–10 fg/cm) to which the termites showed optimal trail-following behavior. The threshold concentration determined in our study for *R. hesperus* was ~60–600-fold lower than previously reported by Matsumura et al. (1972). The difference may be due to the purity of the synthetic dodecatrienol used in the two studies, or to the different substrates on which the trails were laid. In another study, the reported threshold concentration of dodecatrienol for *R. flavipes* was <0.01 pg/cm (Matsumura et al., 1968). On trails of higher concentration (>10 fg/cm), more termites failed to complete the trail or stopped occasionally and reversed direction, resulting in delays to reach the end of the treated line, than did termites on trails with between 1 and <10 fg/cm. Similar results have been reported by other researchers using different termite species (Grace, 1986; Laduguie et al., 1994b; Wobst et al., 1999; Peppuy et al., 2001; Arab et al., 2004; Robert et al., 2004). In our study, this effect manifested itself through a lower percentage of termites completing the trails at higher concentrations within the bioassay time period. Consequently, the walking speed of termites completing the trail was significantly greater for only one concentration (10 fg/cm), and there were no differences in the times taken to complete the trail among the other concentrations. As suggested by Prestwich et al. (1984), such differences may be related to the optimal response behavior of termites to the trail and have less to do with discriminatory ability.

On the Y-maze and the 25-cm long trail, significantly higher numbers of termites responded to 0.1 and 1 fg/cm treatments compared to untreated controls. The minimal threshold concentration of dodecatrienol eliciting trail following was between 0.01 and 0.1 fg/cm. The 1 fg/cm trail remained active for 48 hr. This may be because of the fact that lower concentrations volatilized from the substrate to the point where they were no longer detectable in the bioassay; higher concentrations probably still had some material "loaded" on the substrate. The natural trails and SGE trails (0.2 TE/cm) were active only for 24 hr. Thus, trails have to be reinforced by termites if they are to remain active. Runcie (1987) showed that *R. flavipes* trails leading to a food source were highly persistent compared to exploration trails. Affolter and Leuthold (2000) and Gessner and Leuthold (2001) reached a similar conclusion for trail-following behavior of *Macrotermes subhyalinus* Rambur. For another species, *Nasutitermes costalis*, extracts of 4-yr-old paves, aboveground guidelines formed by feces and debris by foragers, elicited orientation behavior (Traniello, 1982). However, *M. subhyalinus* and *N. costalis* have considerably different foraging and nesting behavior compared to *Reticulitermes* species, and observations on behavior of different species cannot be readily generalized. For example, termites starved for 120 hr were not able to discriminate between different concentrations of dodecatrienol, suggesting that *R.*

hesperus does not use concentration to differentiate between trails leading to food sources or for exploration. Under natural conditions, it seems more likely that the most highly used trails from the colony are those either leading directly to a food source, or foraging trails in a conducive soil environment.

Reticulitermes spp. rarely forage in the open, instead spending most of their time foraging underground in galleries constructed with a number of termites working on each tunnel. In nature, other factors such as tactile stimuli from workers returning from a food source, salivary secretions used to construct tunnels, moisture levels, and the age of the tunnel may provide additional signals.

Reinhard and Kaib (2001) showed that soldiers of *R. santonensis* responded only to trails of higher concentration from SGE of worker termites. In *N. costalis*, worker termites were recruited at higher concentrations compared to soldiers and third instar workers had larger sternal glands compared to soldiers, considered to be responsible for differential recruiting effects (Traniello and Busher, 1985). In our study, different castes of *R. hesperus* (worker, soldier, secondary reproductives, and male alates) responded equally well to dodecatrienol trails at 1 fg/cm. However, at 0.1 fg/cm, soldiers and male alates did not respond significantly more toward the treated arm, and 25% of workers tested failed to complete the 25-cm 0.1 fg/cm dodecatrienol trail.

Differences in threshold concentrations for various trail following chemicals have been reported in various subterranean termite genera and species (Matsumura et al., 1972; Arab et al., 2004). As suggested by other researchers (Howard et al., 1976; Kaib et al., 1982; Tokoro et al., 1994; Cornelius and Bland, 2001), termites of different genera and species may respond to dodecatrienol as a common component of a trail following signal, and there also may be other minor components involved, which may be responsible for species-specific signals.

We chose dodecatrienol concentrations of 1 fg/cm to compare with higher concentrations of dodecatrienol because >90% of the termites were able to follow 1 fg/cm trails. The fact that termites were not able to discriminate between different dodecatrienol concentrations on the two arms of the Y-maze suggests that in nature, trails contain largely qualitative information. Dodecatrienol appeared to act as a repellent at higher concentrations (≥0.01 ng/cm, Table 3). However, when the 0.01-ng/cm concentration was offered simultaneously with 1 fg/cm in a choice assay, termites did not discriminate between either concentration. Furthermore, when offered a choice between dodecatrienol (1 fg/cm) and SGE (1 TE/cm), termites were not able to discriminate between them. This suggests that dodecatrienol is a key component of the SGE signal.

Grace et al. (1988) reported that *R. hesperus* showed a differential response to a concentration gradient of SGE. In contrast, our studies indicated that *R. hesperus* did not respond to a concentration gradient and simply followed the trail. The concentrations selected for our gradient trails were based upon those used in the preliminary straight-line bioassays. Grace (1986) reported that to discriminate between increasing and decreasing gradients the difference on the treated sections must be a little over 10 times. The results suggested that termites showed optimal trail-following behavior at concentrations of between 1 and 10 fg/cm. This range seems more realistic and closer to the concentrations of trails laid by termites in nature. The ability of individual termites to either increase the production of trail pheromone or make it more concentrated so as to provide ≥100-fold differences in deposition on trails in nature seems to be unlikely. In insects such as pharaoh ants, *Monomorium pharaonis*, where trail pheromone is used for trail following, it has been demonstrated that trail geometry rather than a concentration gradient provides polarity to

ant foraging networks (Duncan et al., 2004). Their results showed that bifurcation angles similar to those found in natural networks (~60°) give the best reorientation ratios.

Termites were able to detect the dodecatrienol trails only when their antennae were very close to the trail (about 1–2 mm). The mouthparts apparently did not play a role in detecting the trail and initiating trail following. During normal trail following in termites, maxillary palpi are closer to the substrate than the antennae. When termites were allowed to walk on trail deposits as high as 1 ng/cm of dodecatrienol, they were repelled. In contrast, concentrations as high as 1 ng/cm were not repellent to termites in the bilevel Y-maze in which the antennae and palpi were not directly touching the treated surface. Together, these results suggest that the maxillary palpi may be involved in detecting repellent concentrations of dodecatrienol or perhaps the absence of repellency in the bilevel maze is just because of the additional distance of the antennae from the odor source.

In contrast, arrestant assays showed that male dealates were not repelled by dodecatrienol at relatively high concentrations, with dealates spending more time on treated disks than on controls. In other studies (Laduguie et al., 1994a, b; Robert et al., 2004), it has been suggested that at higher concentrations, dodecatrienol may have another role as a sex pheromone. However, male dealates spent more time on disks treated with extracts of female sternal glands than on disks treated with dodecatrienol, suggesting that other components are probably involved, which may act as synergists with dodecatrienol. On the basis of chemical analysis of extracts of *R. santonensis* (Laduguie et al., 1994a, b), female alates produced 10 to 100 pg of dodecatrienol, approximately 10 times more than the worker termites. Thus, dodecatrienol may be present as one of the components in female sex pheromone, serving to attract male alates for mating at higher concentrations, whereas at lower concentrations, it may act as a trail-following signal, helping in forming tandem pairs during mating.

Earlier studies (Matsumura et al., 1968; Esenther and Beal, 1979; Rust et al., 1996) suggested that wood degraded by *G. trabeum* was attractive to *Reticulitermes* spp., and dodecatrienol was isolated from fungus-degraded wood. Extracts of fungus-degraded wood can be used as arrestants in termite baits and have potential to increase bait station fidelity (Rust et al., 1996). In our feeding studies, we did not observe any phagostimulant or attractant properties of dodecatrienol applied to brown paper towel disks. When termites were transferred to petri dishes in which one half of the disk had been treated with dodecatrienol, they showed greater excitement and movement than termites in untreated controls, and it took longer for them to settle down. Thus, the attractive properties and increased feeding on wood degraded by *G. trabeum* may be due more to the effect of the degradation process on the wood composition or to other as yet unidentified compounds rather than the production of dodecatrienol.

Overall, our studies indicated that the context in which dodecatrienol was presented was critically important. When worker termites were offered a paper disk treated with a spot of dodecatrienol much larger than a termite rather than as a thin line, workers became excited and showed random movements, retouched the spot, and remained in contact with and around the treated disk. Workers perceived the stimulus, but they were not completely arrested by the stimulus. Termites exhibited normal trail-following behavior only when dodecatrienol was presented to them in its normal context, as a thin line. Concentration did not seem to play an important role in routine trail following but may have a role in mating behavior of alates.

Acknowledgments We thank the Carl/Strom Western Exterminator Scholarships for partially funding this research.

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J Chem Ecol (2007) 33:391–404
DOI 10.1007/s10886-006-9231-8

Electrophysiological and Behavioral Responses of *Ips subelongatus* to Semiochemicals from Its Hosts, Non-hosts, and Conspecifics in China

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Received: 25 May 2006 / Accepted: 24 October 2006 /
Published online: 10 January 2007
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Abstract Volatiles from hosts, non-hosts, interspecifics, and conspecifics of the Asian larch bark beetle, *Ips subelongatus* Motsch., were analyzed using both gas chromatographic-electroantennographic detection (GC-EAD) and gas chromatography/mass spectrometry (GC-MS) techniques, and field trapping bioassays in Inner Mongolia, China. GC-EAD experiments indicated that *I. subelongatus* antennae (both sexes) strongly responded not only to the major male-produced conspecific components, ipsenol, and ipsdienol, but also to other bark beetle compounds (*cis*-verbenol and verbenone), host monoterpenes (α-pinene, β-pinene, and para-cymene) from *Larix* sp. logs, and non-host leaf (green leaf volatiles and geranyl acetone) and bark (C_8-alcohols and *trans*-conophthorin) volatiles. Repeatable EAD responses were also found to two compounds from hindgut extracts that are undetectable by GC. One of these minor compounds was identified as amitinol. Field trapping experiments showed that the EAD-active, major male-hindgut component, racemic ipsenol, is the only individual compound that significantly attracted both sexes of *I. subelongatus*, whereas all other compounds, including previously reported pheromone components of European *Ips cembrae*, ipsdienol and 3-methyl-3-buten-1-ol, were unattractive. Ipsdienol, 3-methyl-3-buten-1-ol, or their binary blend had no synergistic or antagonistic effects on *I. subelongatus* attraction when combined with ipsenol, whereas *cis*-

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verbenol (a synomone) and verbenone (the antiaggregation semiochemical) inhibited its attraction to the ipsenol-containing attractive blend. A mixture of three EAD-active host monoterpenes, α-pinene, β-pinene, and para-cymene, was unattractive, but interrupted the pheromone response of *I. subelongatus*. Geranyl acetone, one of the strong EAD-active non-host volatiles also significantly reduced the number of *I. subelongatus* captured in traps baited with ipsenol-containing attractive blend. Our results add support to the recent phylogenetic finding that European and Asian larch bark beetles should be regarded as two distinct species: *I. cembrae* infecting larch in Europe and *I. subelongatus* infesting larch in Asia.

Keywords Coleoptera · Scolytidae · Scolytinae · Aggregation pheromone · GC-EAD · GC-MS · Ipsenol · Ipsdienol · 3-methyl-3-buten-1-ol · Amitinol · *cis*-Verbenol · Verbenone · Geranyl acetone · GLV · NHV

Introduction

The Asian larch bark beetle, *Ips subelongatus* Motsch. 1860, is one of the most serious pest insects in plantations and natural stands of larch (Pinaceae), *Larix sibirica* Ledebour, *Larix gmelinii* (Rupr.) Rupr., *Larixprincipis-rupprechtii* Mayr., *Larix kaempferi* (Lam.) Carr., and *Larix olgensis* Henry in Asia (Zhang et al., 1992). This bark beetle infests weakened, wind-thrown, and burned trees and at high population densities, attacks healthy larch trees (Zhang et al., 1989, 1992). The Asian larch bark beetle has long been considered as *I. subelongatus* by Russian and Chinese entomologists, whereas European colleagues consider this species name a synonym of the European larch bark beetle, *Ips cembrae* Heer 1836 (Postner 1974). However, a recent phylogenetic study by Stauffer et al. (2001) on different populations of the larch bark beetle from Europe and Asia indicated a 4.3% difference in mtDNA sequences between samples from these two regions and suggested that the larch bark beetle complex included at least two taxa: *I. cembrae* infesting larch in Europe and *I. subelongatus* infesting larch in Asia (Stauffer et al., 2001). Therefore, *I. subelongatus* is considered here as a valid species designation for the Asian larch bark beetle.

The aggregation pheromone system of *I. cembrae* was intensively studied in Europe during 1970s to 1980s. A three-component pheromone blend consisting of ipsenol, ipsdienol, and 3-methyl-3-buten-1-ol was first reported by (Stoakley et al., 1978). In the field, the combination of all three components was reported as synergistically attractive (Stoakley et al., 1978). In a later gas chromatography-mass spectrometry (GC-MS) study on aeration samples of *I. cembrae* infested larch logs, another monoterpene alcohol, amitinol, was identified, which significantly enhanced field response to the previously reported aggregation pheromone blend (Kohnle et al., 1988). A commercial lure, Cemprax from Shell Agrar Ltd (Cyanamid GmbH), consisted of ipsenol, ipsdienol, and 3-methyl-3-buten-1-ol has been used to monitor the population dynamics of *I. cembrae* in Germany for some years, but has not shown significant promise for mass-trapping (Zhang and Niemeyer, 1995). Further experiments on field responses to different ratios of the first three components indicated that decrease of 3-methyl-3-buten-1-ol or increase of ipsenol and ipsdienol ratios increased the trap catches (Zhang and Niemeyer, 1995). Unlike the European *I. cembrae*, little is known about the aggregation pheromone system of the Asian *I. subelongatus.* The pheromone biology and colonization behavior of the Asian *I. subelongatus* were studied using natural materials by several Chinese authors in the late 1980s (Fu et al., 1988; Yu and Zhang, 1988; Zhang et al., 1991, 1992). However, it was not until recently that Zhang and coworkers identified several potential pheromone components

from the hindgut samples of the Asian *I. subelongatus* males (still called *I. cembrae* in that paper) by GC-MS analysis, with ipsenol and ipsdienol being the major components and 3-methyl-3-buten-1-ol and several other bark beetle-related compounds as minor components (Zhang et al., 2000). Amitinol, which had been reported as one of the major pheromone components of *I. cembrae* (Kohnle et al., 1988), was not detected in the hindgut samples of *I. subelongatus* males (Zhang et al., 2000). No data are yet available on the electrophysiological and behavioral functions of these male-specific hindgut compounds of Asian larch bark beetles.

Our objectives were to (1) determine electrophysiological responses of the Asian larch bark beetle, *I. subelongatus*, to conspecifically produced compounds (potential pheromone components), interspecific synomones, host volatiles from larch logs, and non-host volatiles from angiosperm trees using GC-electroantennographic detection (EAD)/GC-MS technique and (2) test behavioral activities of the EAD-active compounds in the field in Inner Mongolia, China.

Methods and Materials

Insects Newly emerged *I. subelongatus* adults were collected from naturally attacked *Larix principis-rupprechtii* Mayr. trees in mid-September 2001, at Huamugou Forest Farm (E 117°36′–42′; N 42°24′–52′; 1,500 m a.s.l), Keshiketeng Qi, Inner Mongolia, China. The sexes were separated based on second and third elytral spine differences (Zhang and Niemeyer, 1992). Live beetles (for antennal recordings and solvent extraction of male hindguts) were maintained on newly cut larch logs (*Larix* sp.) and in collection cups with tissue papers in a refrigerator at SLU, Alnarp, Sweden. Ten unfed virgin males were allowed to colonize a 10-cm diameter *Larix* bolt with predrilled holes (25°C, 70% r. h., 20:4 L:D). After feeding for 24 hr, the males were excised from their nuptial chambers, and their hindguts were extracted in 300 μl of hexane.

Headspace Sampling of Fresh Larix Log A freshly cut larch log (10-cm diameter; 30-cm long) was placed into a polyester cooking bag (Meny Toppits, Terinex Ltd, England, 35× 45 cm) equipped with a charcoal filter tube. The headspace volatiles of the log were collected on 30 mg Porapak Q (50–80 mesh, Supelco) in a 3-mm ID Teflon tube via a portable aeration system for 2 hr as described in Zhang et al. (1999) and were extracted with 300 μl of diethyl ether. Both hindgut extracts and aeration samples were kept at −20°C until GC-EAD and GC-MS analyses.

GC-EAD and EAG Responses Two μl of the male hindgut extract and of the *Larix* log aeration sample or 1 μl of synthetic mixtures (ca 100 ng/ul each compound) of host (monoterpenes), non-host (green leaf volatiles and angiosperm bark volatiles) and conspecific compounds were injected splitless into a HP-6890 GC equipped with a HP-INNOWAX column (30 m×0.25 mm×0.25 μm) and a 1:1 effluent splitter that allowed simultaneous flame ionization detection (FID) and EAD of the separated volatile compounds. Hydrogen was used as the carrier gas. The column temperature was 40°C for the first 2 min, rising to 200°C at 10°C/min, and held for 2 min. The outlet for the EAD was inserted into a humidified airstream (0.5 m/s) over an *I. subelongatus* antennal preparation. A glass capillary indifferent electrode filled with Beadle-Ephrussi Ringer, and grounded via a silver wire, was inserted into the severed beetle's, head with antennae while the recording electrode (connected to a high-impedance DC amplifier with automatic

baseline drift compensation) was placed in contact with the distal end of the antennal club. The signal was stored and analyzed on a PC equipped with an IDAC-card and the program EAD ver. 2.3 from Synthech, Hilversum, The Netherlands.

Using the same electrophysiological recording setup and the same antennal preparation technique, EAG dose–responses of *I. subelongtus* to the synthetic 3-methyl-3-buten-1-ol were recorded. This compound was reported as one of three aggregation pheromone components for *I. cembrae* in Europe and was EAD-inactive at the normal dosages from hindgut extracts. Stimuli were prepared by applying 3-methyl-3-buten-1-ol (10^0 to 10^6 ng) in 2 μl of hexane onto a piece of filter paper (7×15 mm) in a Pasteur pipette; the seven concentrations were tested from the lowest to the highest. A solvent blank (2 μl of hexane solvent per filter paper) and an active standard control (100 ng ipsenol per filter paper) stimuli were interspersed between the highest and the lowest dosages of 3-methyl-3-buten-1-ol. Each stimulation was followed by a minimum of 60-sec purge period of filtered air to ensure recovery of antennal receptors. The EAG responses were normalized to the relative response (%) of the active standard control, i.e., 100 ng of ipsenol.

GC-MS Analysis The male hindgut extract and the larch log headspace sample were analyzed with an HP 6890 GC series coupled to an HP 5973 Mass Selective Detector using a DB-WaxETR column (0.25 μm film thickness, 60 m×0.25 mm ID; J and W Scientific, Folsom, CA), programmed from 50°C for the first 2 min, rising to 230°C at 15°C/min, then held for 15 min with helium as carrier gas. Antennally active volatiles were identified by comparison of the retention times and mass spectra with those of the available authentic synthetic compounds and with the computerized data library (Wiley7n.l).

Field Trapping Four field trapping experiments were conducted during the summers of 2002 and 2003 at the same larch plantation stand (ca 26 yr old, 10–11 m high and diameter at breast height=12–14 cm) from where the live *I. subelongatus* adults for EAD recordings and hindgut extraction were collected in 2001. Black flight barrier traps (Theyson, Germany) were used in all four experiments, deployed in lines with 10 m between traps and 30 m between trap lines (experiments). To minimize positional effects, traps together with dispensers within each experiment, were rotated after each replicate (when ≥20 beetles were caught in the best trap) in a randomized Latin-square design (Byers, 1991).

Experiment 1 (20 June–14 July 2002) tested a "natural volatile blend" identified from the hindgut of males (mostly EAD-active) including ipsenol, ipsdienol, 3-methyl-3-buten-1-ol, amitinol, (−)-myrtenol, and 2-phenylethanol, and their one-step deletion blends (subtractive approach), plus the reported pheromone blend for the European larch bark beetle, *I. cembrae*, 3-methyl-3-buten-1-ol, ipsenol, and ipsdienol. Experiment 2 was carried out from 20 June to 14 July 2002 to determine the behavioral activity of the three reported pheromone components, 3-methyl-3-buten-1-ol, ipsenol, or ipsdienol, and all their possible binary and ternary blends. Experiment 3, conducted at the same location and time period as the previous two, focused on potential effects of EAD-active compounds from conspecifics, hosts, and non-hosts on bark beetle attraction to the ternary blend of 3-methyl-3-buten-1-ol, ipsenol, and ipsdienol. A mixture of three EAD-active host monoterpenes was also included in the experiment because these had not been previously tested. Experiment 4 was carried out from 30 May to 5 July 2003, when all the treatments in experiment 2 were repeated, plus three additional treatments, including 2-phenylethanol alone, the combination of ipsenol and 2-phenylethanol, and a blank control. All chemicals, sources, release devices, and release rates are listed in Table 1.

Table 1 Chemicals, release rates, and dispensers used in field trapping experiments

Chemical	Source	Purity (%)	Release Rate (mg/24 hr)[a]	Loading (μl/vial)	Dispensers[b]
(±)-Ipsenol (Ie)	Borregaard , Norway	>98	1.4	20	Two open #730 PE vials
(±)-Ipsdienol (Id)	ICN Biomedicals, USA	95	0.6	20	One open #730 PE vial
3-Methyl-3-buten-1-ol (MB)	Aldrich Chemical Co.	>97	15	500	One open #730 PE vial
Amitinol (At)	W. Francke, Hamburg Univ., Germany	98	1	20	One open #730 PE vial
(−)-Myrtenol (Mt)	Acros Organics	97	0.5	50	One open #730 PE vial
2-Phenylethanol (PE)	Aldrich Chemical Co.	99	0.5	50	One open #730 PE vial
cis-Verbenol (cV)	J. Vrkoè, IOBC, Prague, Czechia	98	0.5	30	Two open #730 PE vials
(−)-Verbenone (Vn)	Bedoukian Research Inc.	99	0.5	200	One open #730 PE vial
Geranyl acetone (GA)	Aldrich Chemical Co.	98	0.2	75	Two open #730 PE vials
Mixture of 3 monoterpenes			40	1,025	Two open 2-ml glass vials
α-Pinene	Aldrich Chemical Co.	98		750	
β-Pinene	Aldrich Chemical Co.	99		250	
para-Cymene	Aldrich Chemical Co.	99		25	

[a] Measured in a mini wind-tunnel at 20–21°C and 0.7 m/sec for 1 week

[b] PE-vial with 6-mm diameter opening, 29-mm inner length

Statistical Analysis Trap catch data were transformed by log $(x+1)$ to fit the assumption of homogeneity of variance for analysis of variance (ANOVA). The means were compared by ANOVA followed by the Ryan–Einot–Gabriel–Welsh (REGW) multiple Q test (SPSS 8.0 for Windows) at $\alpha=0.05$. The sex ratios of beetles captured for the treatments within each experiments were compared with 95% binomial confidence intervals (Byers and Wood, 1980).

Results

GC-EAD Recordings and EAG Dose–Responses GC-EAD/MS analyses on the hindgut extract of males indicated that ipsenol and ipsdienol are the major components (ca 3:1 ratio of ipsenol to ipsdienol) eliciting very strong EAD responses by both sexes (Fig. 1a). No antennal responses were detected to the minor components, 3-methyl-3-buten-1-ol, geraniol and 2-phenylethanol at the natural dosage from the extract; however, repeatable EAD responses to two FID undetected compounds (amounts under GC-FID/MS detectable levels) were recorded. Based on the retention time and a further EAD analysis on a synthetic mixture (Fig. 1a), one of the FID undetected, antennally active compounds was

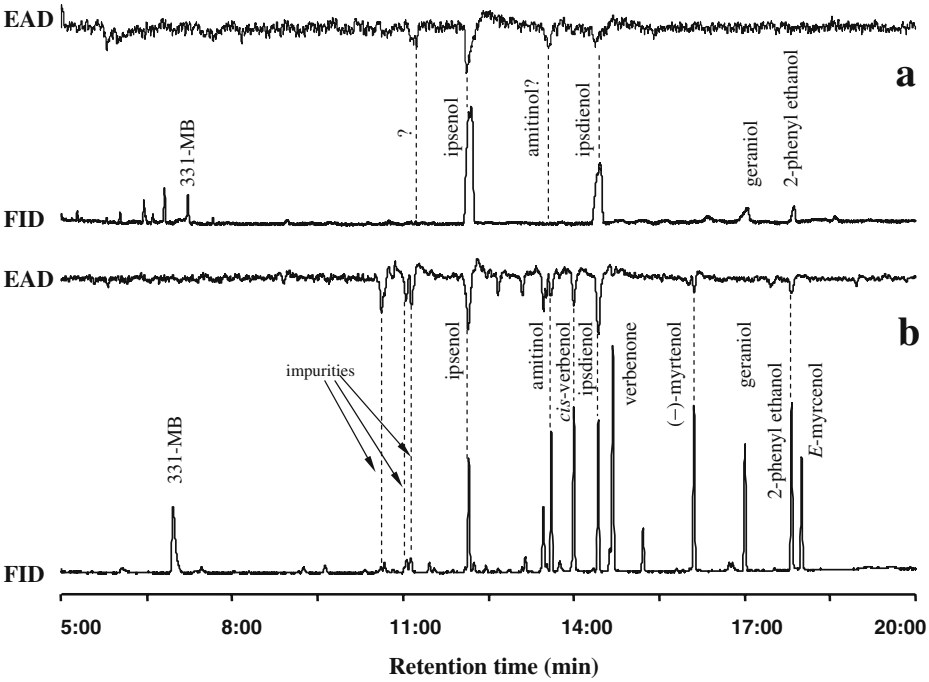


Fig. 1 GC-EAD responses of *Ips subelongatus* antennae to (**a**) hindgut extract of males feeding for 24 hr under the bark of *Larix* logs; ipsenol was ca 20 ng; (**b**) synthetic mixture including 10 major bark beetle related semiochemicals (ca 50 ng/compound); EAD active impurities (probably from ipsenol) were not identified. *Vertical lines* (- - - -) connect FID peaks with repeatable EAD peaks

tentatively identified as amitinol, whereas the other active compound remains unknown. EAD analysis on a synthetic mixture of bark beetle related compounds not only confirmed the strong antennal activities to ipsenol, ipsdienol, and amitinol from the hindgut extract of males, but also showed a significant EAD response to *cis*-verbenol, one of the major aggregation pheromone components of *Ips typographus* L. and several other *Ips* bark beetles (Byers 2004) (Fig. 1b). At the dosage tested (50 ng exposed to the antennal preparation after FID and EAD split), *I. subelongatus* antennae also responded to the minor or trace components of the hindgut extract, (−)-myrtenol and 2-phenylethanol (Fig. 1b), whereas verbenone, an antiaggregation semiochemical for many *Ips* bark beetles, was only weakly active. 3-Methyl-3-buten-1-ol and geraniol, the minor components from the male hindgut extracts and one of the major pheromone components of *I. duplicatus* (Sahlb.), *E*-myrcenol, were antennally inactive (Fig. 1b). In addition, there were some repeatable EAD responses to several unidentified impurities of the synthetic mixture (Fig. 1b). EAD analyses on the headspace sample of the fresh larch log showed strong antennal responses to two major monoterpene hydrocarbons, α-pinene and β-pinene; repeatable responses were also recorded to two minor components, para-cymene and 1-hexanol (Fig. 2). The identities of these EAD active compounds were identified by GC-MS and confirmed by GC-EAD of synthetic mixtures (results not shown). Further GC-EAD analyses on synthetic mixtures of non-host leaf and bark volatiles showed significant antennal responses to green leaf alcohols (C_6-alcohols), linalool, and geranyl acetone from angiosperm leaves, and *E*-conophthorin, C_8-alcohols and benzyl alcohol from the non-host bark (Fig. 3, 4). Other

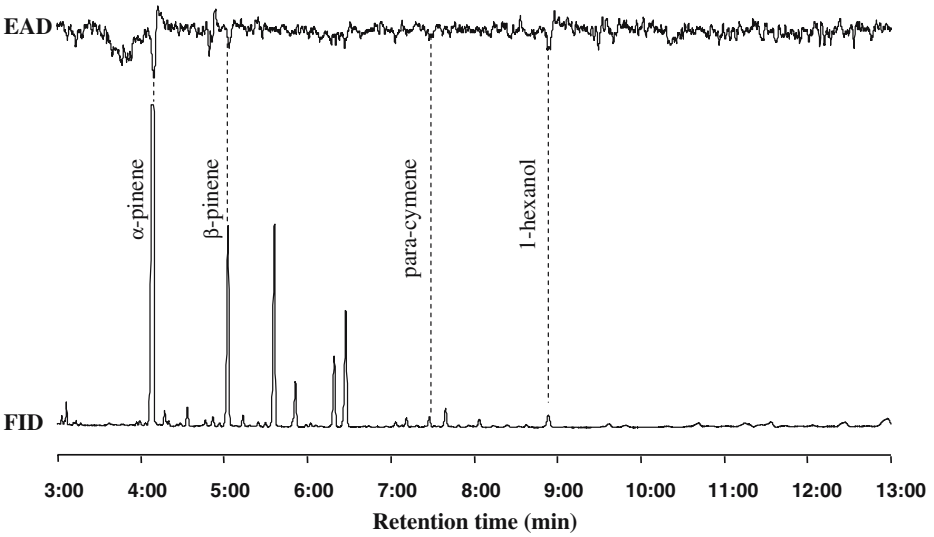


Fig. 2 GC-EAD responses of *Ips subelongatus* antennae to aeration sample of fresh *Larix* sp. host log (α-pinene was ca 140 ng)

common volatiles from angiosperm trees, such as C_6-aldehydes, benzyaldehyde, salicylaldehyde, and *Z*-3-hexenyl acetate were inactive at the dosage tested (Fig. 3, 4). Geranyl acetone and *E*-conophthorin seemed to elicit larger EAD responses than did the other non-host volatiles. No differences in antennal responses between males and females were observed. Results from EAG dose–responses to 3-methyl-3-buten-1-ol indicated that this compound is simply inactive at loading dosages of 10 μg or lower. A weak EAG response was recorded to 100 μg of 3-methyl-3-buten-1-ol, whereas significant antennal reaction was

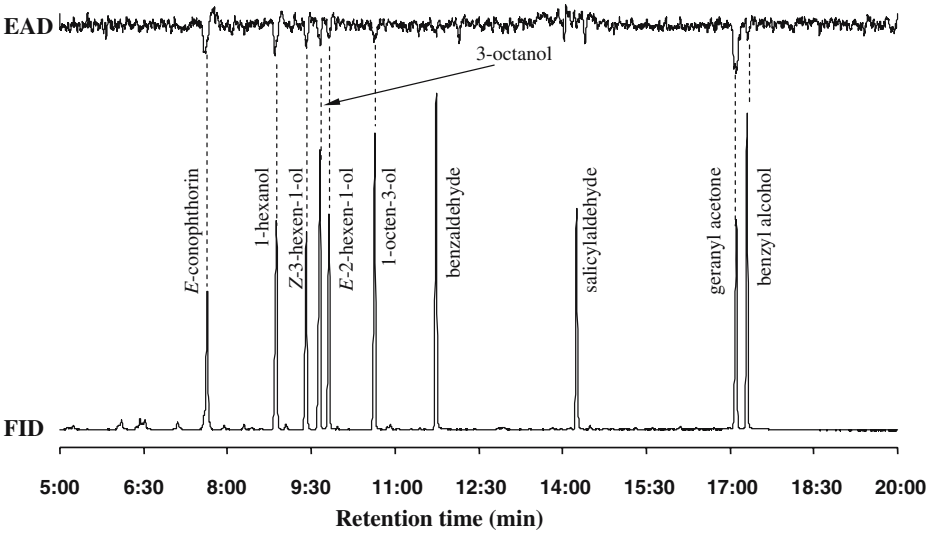


Fig. 3 GC-EAD responses of *Ips subelongatus* antennae to a synthetic mixture of ten non-host leaf and bark volatiles (50 ng/compound). *Vertical lines* (- - - -) connect FID peaks with repeatable EAD peaks

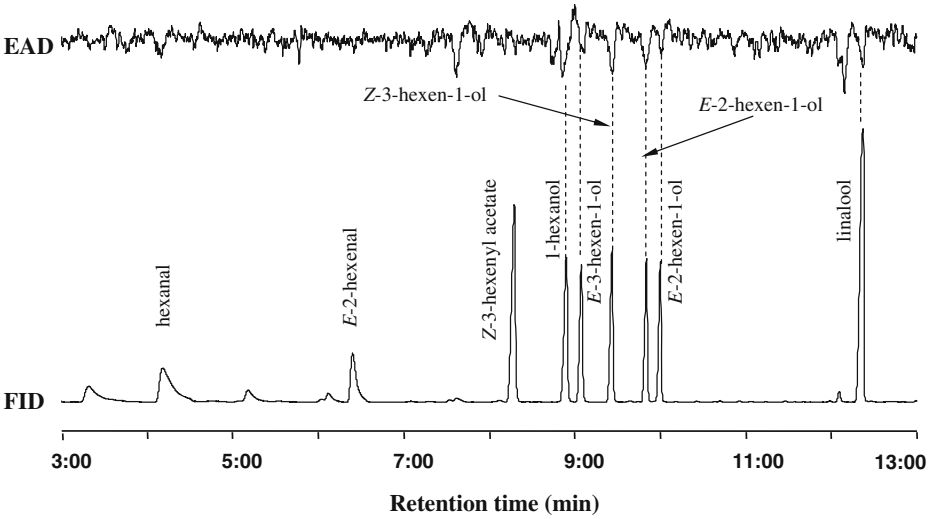


Fig. 4 GC-EAD responses of *Ips subelongatus* antennae to a synthetic mixture of several green leaf volatiles plus linalool (50 ng/compound). *Vertical lines* (- - - -) connect FID peaks with repeatable EAD peaks

observed only at 1 mg (Fig. 5). The EAG response threshold to 3-methyl-3-buten-1-ol seemed to be at least 10,000 times higher than the active standard ipsenol.

Field Trapping In experiment 1, carried out in 2002, a total of 1,153 beetles were caught during 8 replicates. The "natural volatile blend" containing five EAD-active compounds

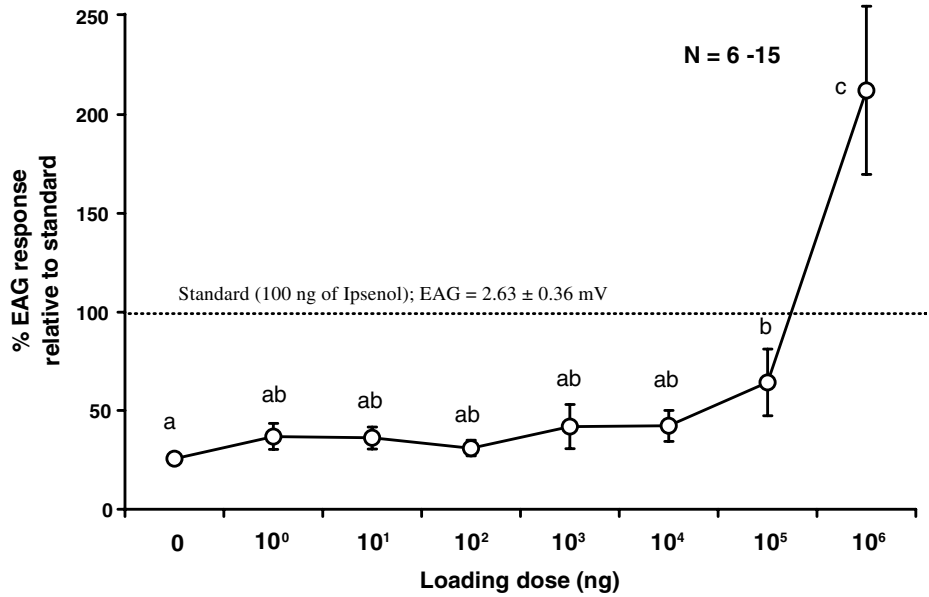


Fig. 5 EAG dose–responses of *Ips subelongatus* antennae to 3-methyl-3-buten-1-ol, one of the reported aggregation pheromone components for this species

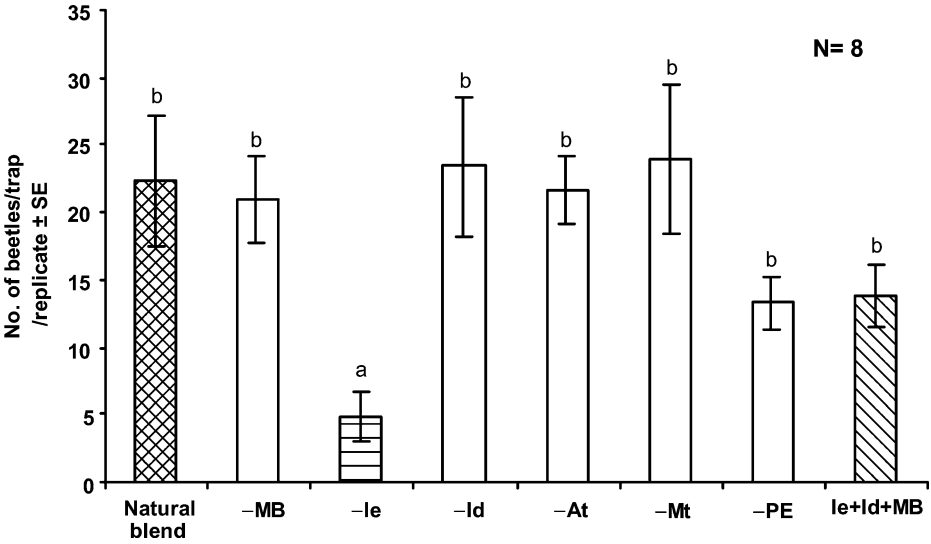


Fig. 6 Subtractive field assay of *Ips subelongatus* in traps baited with a full natural blend [3-methyl-3-buten-1-ol (*MB*), ipsenol (*Ie*), ipsdienol (*Id*), amitinol (*At*), (−)-myrtenol (*Mt*) and 2-phenylethanol (*PE*); mostly EAD-active] and partial blends; Keshiketeng Qi, Inner Mongolia, China, June 20–July 14, 2002. Means followed by the same letter are not significantly different (ANOVA on Log(x+1), REGW multiple Q-test, P>0.05)

plus 3-methyl-3-buten-1-ol identified from the male hindgut extracts was not significantly different from the mixture of the three reported pheromone components for the European *I. cembrae*, ipsenol, ipsdienol, and 3-methyl-3-buten-1-ol. Subtraction of the major component, ipsenol, from the "full blend" significantly reduced the trap catches by 78%, whereas removal of other components had no effect (Fig. 6). There were no significant differences in sex ratios (♀:♂=2.99±0.31:1) among treatments. In experiment 2, a total of 328 beetles were captured during 7 replicates with a mean sex ratio (♀:♂) of ca 2.91± 0.32:1 (no differences among treatments; 95% binomial CI). 3-Methyl-3-buten-1-ol, ipsdienol, or their combination was unattractive at the dosage tested, whereas ipsenol alone was significantly attractive to both sexes of *I. subelongatus*. Ipsenol-containing binary and ternary blends were also attractive, but were not different from the ipsenol (Fig. 7). In experiment 3, 154 beetles were recorded in 7 replicates with a mean sex ratio (♀:♂) of ca 3.12±0.59:1 (no differences among treatments; 95% binomial CI). A mixture of three EAD-active host monoterpenes, α-pinene, β-pinene, and para-cymene was not active at the dosage tested, whereas the mixture of three reported aggregation pheromone components, ipsenol, ipsdienol, and 3-methyl-3-buten-1-ol was attractive (Fig. 8). Addition of amitinol (EAD-active, FID undetected male hindgut extracts) slightly increased the trap catch, but was not statistically significant. Interestingly, addition of either the host monoterpene mixture, *cis*-verbenol, verbenone, or the non-host volatile, geranyl acetone to the active mixture significantly reduced the numbers of *I. subelongatus* captured (Fig. 8). The inhibitory effect by *cis*-verbenol was stronger than that of the other compounds. In experiment 4 carried out in 2003, a total of 1,489 beetles were trapped in 10 replicates with a mean sex ratio (♀:♂) of ca 2.1±0.42:1 (no differences among treatments; 95% binomial CI). Similar to experiment 2, 3-methyl-3-buten-1-ol, ipsdienol, or their combination was

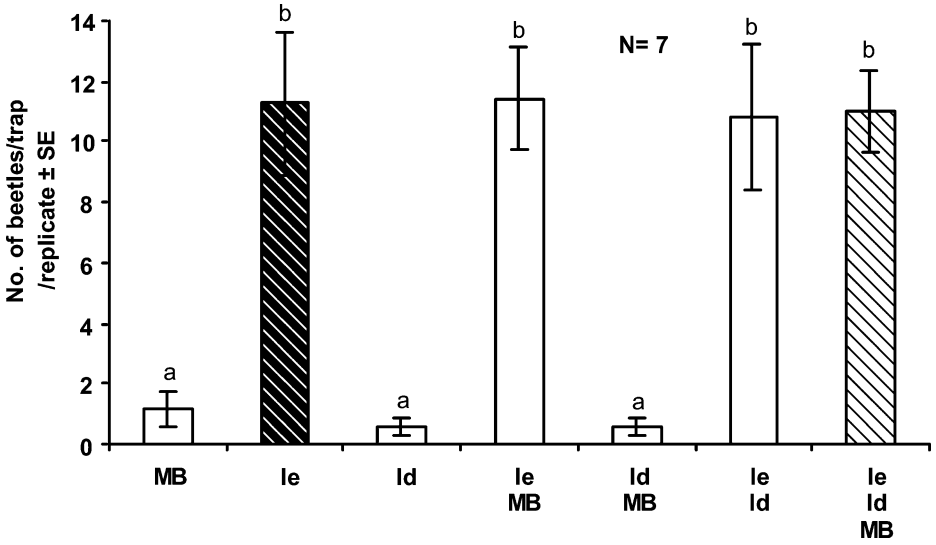


Fig. 7 Full factorial field assay of *Ips subelongatus* in traps baited with reported pheromone components of *I. cembrae*; 3-methyl-3-buten-1-ol (*MB*), ipsenol (*Ie*), or ipsdienol (*Id*) and their binary and ternary blends, Keshiketeng Qi, Inner Mongolia, China, June 20–July 14, 2002. Means followed by the same letter are not significantly different (ANOVA on Log(x+1), REGW multiple Q-test, P>0.05)

simply inactive, whereas ipsenol alone was strongly attractive (Fig. 9). Addition of 3-methyl-3-buten-1-ol, or ipsenol, or both to ipsenol did not increase trap catches. 2-Phenylethanol alone was unattractive and showed no effects on attraction of *I. subelongatus* when added to ipsenol (Fig. 9).

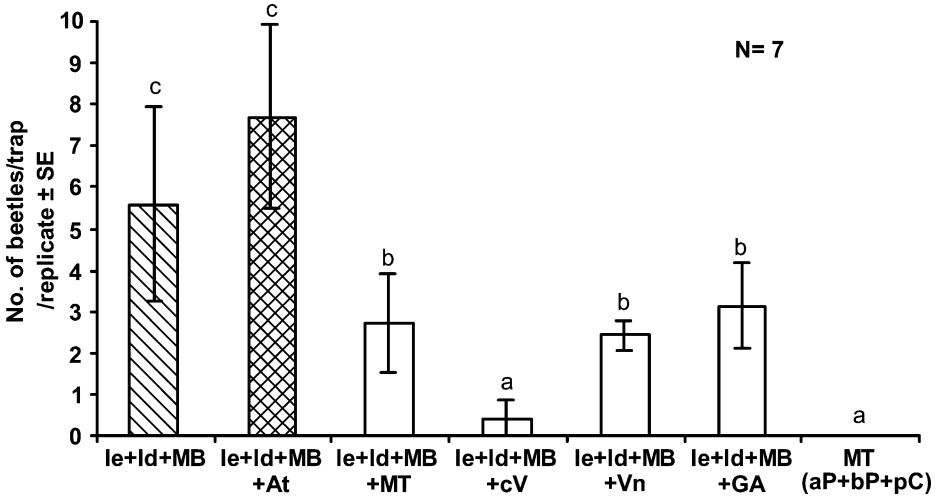


Fig. 8 Additive field assay of five EAD active compounds found from conspecifics, host and non-hosts of *Ips subelongatus* in traps baited with a mixture of three major male-produced compounds: 3-methyl-3-buten-1-ol (*MB*), ipsenol (*Ie*), and ipsdienol (*Id*), and a mixture of three monoterpenes (MT): α-pinene (aP), β-pinene (bP), and para-cymene (pC) was also tested, Keshiketeng Qi, Inner Mongolia, China, June 20–July 14, 2002. Means followed by the same letter are not significantly different (ANOVA on Log(x+1), REGW multiple Q-test, P>0.05)

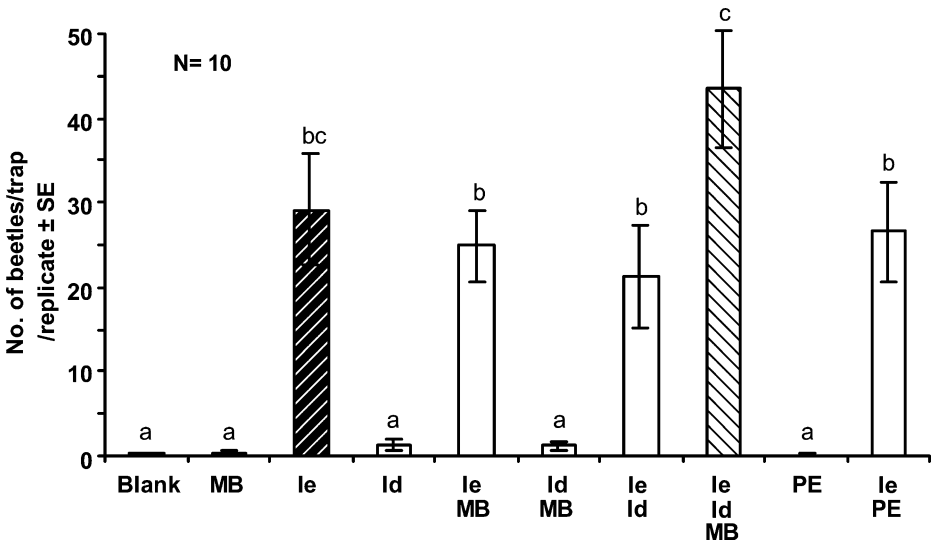


Fig. 9 Full factorial field assay of *Ips subelongatus* in traps baited with the reported pheromone components of *I. cembrae* and their binary and ternary blends, plus 2-phenylethanol (PE) and its combination with ipsenol, Keshiketeng Qi, Inner Mongolia, China, May 30–July 5, 2003. Means followed by the same letter are not significantly different (ANOVA on Log (x+1), REGW multiple Q-test, P>0.05)

Discussion

This is the first detailed electrophysiological study on semiochemicals of the Asian larch bark beetle, *I. subelongatus.* Previously, however, we did report on the enantiospecific antennal responses of several *Ips* bark beetles including *I. subelongatus* (EAD dose–response) to (5*S*,7*S*)-conophthorin, one of the major non-host volatiles from angiosperm trees (Zhang et al., 2002). Present GC-EAD analyses revealed that *I. subelongatus* antennae strongly responded to the male-produced conspecific components, ipsenol, ipsdienol, and two FID undetected compounds from male hindgut extracts, aggregation pheromone components of other *Ips* bark beetles (e.g., *cis*-verbenol), monoterpenes (α-pinene, β-pinene, and para-cymene) and 1-hexanol from host logs (*Larix* sp.), and non-host leaf (i.e., GLVs and geranyl acetone), and non-host bark (i.e., C_8-alcohols and *trans*-conophthorin) volatiles. One of the EAD-active, FID-undetected compounds was tentatively identified as amitinol, whereas the other one remains unknown. Amitinol is a pheromone component of *Ips aminitus* (Eichhoff) (Francke et al., 1980), *Ips tridens* Mannerheim (Poland and Borden, 1997), and *I. cembrae* (Kohnle et al., 1988). Similarly, amitinol was not detected by GC-FID/MSD from *Ips duplicatus* (male hindgut extracts and aeration samples of male-colonized spruce logs), but was identified with the aid of GC-EAD technique, and shown to be a potential aggregation pheromone component for this bark beetle at low release rate (Zhang et al., 2007).

Our field tests showed that ipsenol is the only individual compound that significantly attracts both sexes of *I. subelongatus*, while all other compounds (individuals or in combinations) were unattractive. These results indicate that ipsenol is the key, if not the sole, aggregation pheromone component for the Asian larch bark beetle.

For the European *I. cembrae*, ipsdienol and 3-methyl-3-buten-1-ol were reported as parts of a synergistic mixture together with ipsenol, forming a three-component aggregation

pheromone system (Stoakley et al., 1978; Renwick and Dickens, 1979). It should be noted, however, that the published data in support of the synergistic activity of 3-methyl-3-buten-1-ol is weak. On the other hand, when ipsdienol was left out of the ternary blend, the attraction of *I. cembrae* was totally eliminated (Stoakley et al., 1978). In the current study, the functionality of these two compounds as pheromone components of *I. subelongatus* is not supported. Addition of amitinol at relatively high release to the ternary blend of ipsenol, ipsdienol, and 3-methyl-3-buten-1-ol increased trap catches but not significantly. A weak increase in *I. cembrae* trap catches was also observed in Germany (Zhang and Niemeyer, 1995), whereas Kohnle et al. (1988) indicated a significant enhancement of trap catches with the addition of amitinol at high release to the ternary pheromone blend. The disparate results between these two studies might be due to different amitinol release rates used. In a recent study, addition of a small amount of amitinol to a blend of ipsdienol and (*E*)-myrcenol significantly increased the trap catches of *I. duplicatus* by 47%, whereas addition of amitinol at the high release had no positive or negative effects on the trap catches (Zhang et al., 2007). Another EAD-active and FID undetected compound from the hindgut extracts of *I. subelongatus* males remains unidentified; therefore, its functionality as a potential synergistic aggregation pheromone component cannot be excluded. The natural enantiomeric compositions of ipsenol and ipsdienol from Asian larch bark beetles (*I. subelongatus*) were 100% (*S*)-(−)-ipsenol and 96% (*S*)-(+)-ipsdienol (Zhang et al., 2000), similar to those found from the European *I. cembrae* (Francke and Vite, 1983). The inactivity of racemic ipsdienol in the current study might be due to an inhibitory effect of the unnatural antipode in the synthetic samples. However, no such inhibitory effect was indicated in the synergistic attraction of *I. cembrae* by racemic ipsdienol (Stoakley et al., 1978). On the other hand, we do not know if pure (*S*)-(−)-ipsenol would attract significantly more *I. subelongatus* than did the racemic ipsenol. To fully understand the aggregation pheromone system of Asian *I. subelongatus*, more work is needed to (1) identify the new FID-undetected/EAD-active compound, and (2) field test all the EAD-active compounds including the enantiomerically pure samples of ipsenol and ipsdienol.

cis-Verbenol is one of the two major aggregation pheromone components of the spruce bark beetle, *I. typographus* (Bakke 1976; Schlyter et al., 1987), and this compound virtually blocked the attraction of *I. subelongatus* to the ipsenol-containing attractive blend. Such an inhibitory effect was also reported for the European *I. cembrae* by Kohnle et al. (1988), which might function as an interspecific synomone between these sympatric and closely related species. Verbenone has been shown to inhibit response in over 10 species of conifer bark beetles to attractant-baited traps and trees and to deter attack in the treated conifer stands (see Borden 1997 for details). It also showed a significant inhibitory effect on attraction of *I. subelongatus* to the ipsenol-containing attractive blend. A mixture of the three EAD-active host monoterpenes, α-pinene, β-pinene, and para-cymene, was unattractive to *I. subelongatus*, but instead, the mixture inhibited the attraction to the ipsenol-containing attractive blend at the dose tested. This result suggests no long-range primary attraction to these host volatiles and no positive effect of these host volatiles to the aggregation pheromone in *I. subelongatus*. Addition of α-pinene (Stoakley et al., 1978) and larch logs (Zhang and Niemeyer, 1995) to the aggregation pheromone mixture also did not increase trap catches for *I. cembrae*. Our data and earlier reports support the hypothesis that primary attraction to host volatiles is absent (or very weak) in aggressive bark beetle species, especially *Ips* spp with strong aggregation pheromones (Schlyter and Birgersson, 1999). The inhibitory effect observed in our study might be due to a high release rate of the monoterpenes, which in nature, may be indicative of a vigorous and healthy tree unsuitable

for the bark beetle attack. Geranyl acetone, one of EAD-active non-host volatiles, significantly reduced the numbers of *I. subelongatus* captured when added to the ipsenol-containing attractive blend. Other EAD-active non-host volatiles were not tested in the current study, but their strong inhibitory effect on many other conifer bark beetles (see Zhang and Schlyter, 2004 for details) might suggest a similar function on *I. subelongatus* as well.

In summary, the most striking result of the present investigation was the discovery that *I. cembrae* and *I. subelongatus* respond differently to ipsdienol and 3-methyl-3-buten-1-ol. This result adds new support to the recent phylogenetic finding (Stauffer et al., 2001) that European *I. cembrae* and Asian *I. subelongatus* larch bark beetles are best treated as two distinct species. However, reexamination of the European *I. cembrae* samples with modern techniques such as GC-EAD might provide new understanding of its aggregation pheromone system (Sullivan 2005). From the economical point of view, traps baited with racemic ipsenol alone might be adequate for the purpose of monitoring *I. subelongatus* populations, while a natural synergistic aggregation pheromone blend, including ipsenol and other unidentified (missing) components, may have a stronger potential for mass trapping.

Acknowledgements We thank Zhang, Minghua and Wei, Yanjun from Forest Pest Control Station of Keshiketeng Qi, Inner Mongolia, China for field assistance, and Prof. Wittko Francke, University of Hamburg, Germany for the synthesis of amitinol. Valuable comments by Dr. Jeff. R. Aldrich, CAIBL/USDA/ARS, Beltsville, USA are appreciated. This study was conducted while the first author was supported by grants from the Swedish Council for Forestry and Agricultural Research (SJFR: 24.0293/98), FORMAS (230-2005-1778), and an EU 4fp project ("TATRY", CT 98-0151).

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J Chem Ecol (2007) 33:405–415
DOI 10.1007/s10886-006-9224-7

The Male Abdominal Glands of *Leucophaea maderae*: Chemical Identification of the Volatile Secretion and Sex Pheromone Function

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Received: 26 July 2006 / Revised: 4 October 2006 / Accepted: 30 October 2006 /
Published online: 3 January 2007
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Abstract In *Leucophaea maderae*, male calling behavior involves the release of a sex pheromone from the abdominal sternal glands. An extract of sternal glands attracted conspecific females over a distance. The compounds present were identified as hydroxy-3-butan-2-one, (2*R*, 3*R*)-butanediol, senecioic acid, and (*E*)-2-octenoic acid. The same components are also present in male tergal glands. The identified compounds were tested on their own and in mixtures. Their biological function is discussed.

Keywords Cockroach · Dictyoptera · Blaberidae · *Leucophaea maderae* · Hydroxy-3-butan-2-one · (2,3)-Butanediol · 3-Methyl-2-butenoic acid · Senecioic acid · (*E*)-2-Octenoic acid · Sex pheromone · Sexual behavior

Introduction

In their observations on the mating behavior of *Leucophaea maderae*, Roth and Willis (1954) and Roth and Barth (1964) noted that virgin females did not seem to produce any sex pheromones that attracted males over a distance. A few years later, Barth (1968) found that the whole extracts of males were attractive to conspecific females. Later, Sreng (1984) showed that the male adopts a characteristic calling behavior and emits a sex pheromone from the sternal abdominal glands, which attracts females over a distance. Once both sexes are in antennal contact, the male raises its wings, revealing its tergal glands, which are covered with proteinaceous secretions (Korchi et al., 1998). The female then climbs onto the male's back to feed on this secretion. As a consequence of this behavior, the female is in the proper position for copulation.

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Male volatile pheromones that function as attractants for females are relatively rare among cockroaches. The Madera cockroach, *L. maderae*, and the lobster cockroach, *Nauphoeta cinerea*, belong to the Oxyhaloinae, a small subfamily in which mating behavior is initiated by the emission of a male volatile sex pheromone secreted by the sternal glands (Sreng, 1979, 1984). The composition of the pheromone of *N. cinerea* was elucidated in our laboratory (Sreng, 1990; Sirugue et al., 1992). It is a multicomponent blend with three biologically active compounds: 2-methylthiazolidine, 4-ethylguaiacol, and hydroxy-3-butan-2-one. This secretion may also function in sexual selection and mate choice by the female (Smith and Breed, 1982; Schal and Bell, 1983; Moore, 1989, 1990, 1997; Moore and Moore, 1999; Moore et al., 2001).

In this paper, we report on the identification of compounds in the male tergal and sternal glands of *L. maderae* and the behavioral role of the identified compounds. These results are compared to those obtained from *N. cinerea*.

Methods and Materials

Insect Colonies of cockroaches were reared in glass aquaria (40×25×25 cm) maintained at a constant temperature of 26±2°C and 70% relative humidity under a reversed 12:12 photoperiodic cycle. Dry dog food and water were provided ad libitum.

Adults of both sexes, used in the bioassays, were randomly isolated from stock colonies at adult ecdysis. To avoid any mechanical or olfactory stimuli between the sexes, males and females were isolated in plastic boxes (9×6×2 cm) containing food and water. Each sex was kept in a separate room under the same rearing conditions.

Preparation of Extracts Isolated males and females (20 days old) were cooled for a few minutes at −20°C before being used 2–3 h after the beginning of the dark period. This age corresponds to the sexual maturity of adult males and females, whereas the diel period for testing corresponds to peak male calling activity of this species (Sirugue, 1992).

For gas chromatographic (GC) analyses and quantification, all the abdominal sternites and tergites of each adult were dissected rapidly with microscissors and forceps. After removing the adjacent tissues (fat body, hemolymph) with a paper towel, the sternites and tergites were placed individually into vials containing 4 ml of distilled CH_2Cl_2 and 10 μg of 2-phenylethanol as an internal standard. The tissue was allowed to extract for 3 h at room temperature, after which it was removed, and 500 μl of the extract decanted into a 2-ml vial. Extracts were kept at −20°C until analyzed.

Chemical Analysis GC analyses were performed with a CP 9000 Chrompack GC fitted with a flame ionization detector. A SPB™1701 fused silica capillary column (30 m×0.25 mm ID, 0.25 μm film thickness, Supelco, Bellefonte, PA, USA) was used for analyses. After concentration of the extract to about 10 μl, by gentle nitrogen flow, 2 μl of sample was injected into an injector operating in the splitless mode. The column was held isothermally at 40°C for 2 min, then programmed at the rate of 20°C/min for 1 min, 2°C/min to 150°C, and then 10°C/min to 250°C. Hydrogen was used as carrier gas (50 cm/s velocity at ambient temperature). The injector and detector temperatures were 260 and 280°C, respectively.

The stereochemical configuration of 2,3-butanediol was determined by retention time comparisons of the authentic standards on a Beta Dex 120 fused silica capillary chiral column (30 m × 0.25 mm ID, 0.25 μm film thickness, Supelco). GC conditions were as

previously described except that the column oven was programmed from 40 to 220°C at a rate of 5°C/min.

Gas chromatography-mass spectrometry was carried out with a Nermag R 10-10 C quadrupole mass spectrometer coupled with a Hewlett-Packard 5890 GC. The GC conditions were the same as those described above. The column was connected directly to the ion source of the spectrometer through a heated transfer line maintained at 260°C. Electron impact mass spectra were obtained at 70 eV with a 0.8-s cycle, with a source temperature of 150°C.

Compounds were identified by comparing spectra to those in the EPA/NIH (Heller and Milne, 1978), NIST/EPA/MSDC (Lias and Stein, 1990), and FLAVIC laboratory (INRA, Dijon, France) libraries. Identification of all compounds was performed by analyzing the natural products and their synthetic counterparts under the same conditions on the SPB™1701 column. The detector response factor was determined for each of the identified compounds by using 0.5, 1, 5, and 10 μg of pure chemical and used in the quantitative analyses of 10 individual males and 10 individual females.

Commercial Compounds Hydroxy-3-butan-2-one (acetoin), (2*R*, 3*R*)- and (2*R*, 3*S*)-butanediol were a gift from INRA (Dijon, France); (2*S*,3*S*)-butanediol was bought from Extrasynthèse (Geray, France), 2-phenylethanol and (*E*)-2-octenoic acid from Interchim (Montluçon, France), and 3-methyl-2-butenoic acid (senecioic acid) from Ega-Chemie (Lyon, France). All chemicals used were greater than 98% purity as determined by capillary GC analysis.

Bioassays (Y-maze olfactometer) The olfactometer was a glass tube (2 cm ID.) with a 40-cm-long common arm, dividing into two 30-cm-long choice arms, A and B (70° angle between the two choice arms). Individuals were placed separately in a test box (6×9×5 cm) for at least 2 h before each test. The box had a screen door that could be raised to allow the insect to enter the common arm at the starting point. An air stream (10 ml/s) conveyed the chemical stimulus through arm A or B to the common arm and to the "starting box" containing the insect. Each insect was tested once for 5 min. If an insect did not respond within this period, the test was stopped. The olfactometer was cleaned with CH_2Cl_2 after each test. All observations were performed between the second hour and sixth hour of the scotophase under a dim red light. Which of the two test arms the insect entered was recorded, or if the insect did not enter the common arm of the olfactometer, it was recorded as a nonresponder. The data were compared by using a binomial test according to Parker (1979). Each test was replicated 30 times using different insects.

In order to prove the existence of a male sex pheromone that attracts females over a distance, various olfactory stimuli were tested on sexually mature virgin females (20 days old): one mature virgin male (20 days old)/air, one mature virgin female (20 days old)/air, and various concentrations of male sternal gland extracts (1 to 10^{-3} male equivalent)/air. A control test (2 μl CH_2Cl_2/air) was carried out in order to check that there was no bias by the insects to one of the arms when no stimulus was present. The chemical extracts were deposited on a folded filter paper (2 cm^2), the solvent was allowed to evaporate, and the filter paper was placed at the opening of one of the choice arms (alternatively, A or B). Because the tergal glands are not involved in long-range attraction (Sreng, 1984), and their chemical composition is identical to that of the sternal secretion (see results), their extract was not tested.

In a second series of experiments, we tested various concentrations of the compounds. The experiment was conducted as described above. First, each component was tested

individually at the following concentrations: hydroxy-3-butan-2-one (H3B2) (0.1, 0.5, 1, 5, 10, 100, and 500 ng, 1 μg), (2*R,* 3*R*)-butanediol [(2,3)-BUT)] (0.1, 10, 100, 150, and 400 ng, 1 μg), senecioic acid (SENE Ac) (0.1, 1, 10, and 100 ng, 1, 2.8, and 7 μg), and (*E*)-2-octenoic acid [(*E*)-2-OCT Ac] (0.1, 1, 10, 100, 200, and 100 ng). Second, various mixtures, based on the concentration of each component in 10^{-1} male equivalent (10^{-1} ME; 20 days old) were tested: H3B2/(2,3)-BUT (380/160 ng), H3B2/SENE Ac (380/2,800 ng), H3B2/(*E*)-2-OCT Ac (380/100 ng), and H3B2/(2,3)-BUT/SENE Ac/(*E*)-2-OCT Ac (380/160/2,800/100 ng).

Results

Chemical Identification A typical GC trace of the sternal gland extract of adult males revealed the presence of four major components, which represented more than 95% of the volatile portion of the extract (Fig. 1). Comparable GC traces were obtained from male tergal gland extract. None of these compounds was detected in tergal and sternal gland extracts of females although a number of minor, unidentified peaks were common to both male and female extracts. Based on their mass spectra, the four major compounds in male extracts were identified as hydroxy-3-butan-2-one (MW 88), (2, 3)-butanediol (MW 90), 3-methyl-2-butenoic acid (senecioic acid; MW 100), and (*E*)-2-octenoic acid (MW 142). (2, 3)-Butanediol consisted of more than 99% of the (2, 3) enantiomer, and only traces of the other enantiomeric forms of the molecule were found (see methods).

The amount of each compound (mean±SEM) in the extract varied with individuals and glandular origin. In the sternal glands, all compounds were abundant: hydroxy-3-butan-2-one (3,794±239 ng), (2*R*, 3*R*)-butanediol (1,626±93 ng), senecioic acid (28,209±613 ng), and (*E*)-2-octenoic acid (1,002±22 ng). In the tergal glands, the compounds were found in

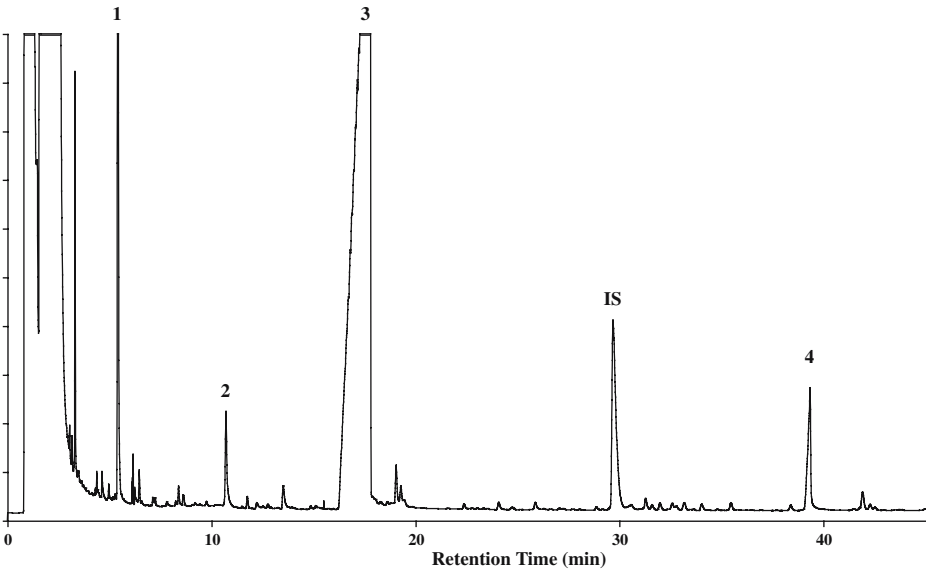


Fig. 1 Typical GC trace with identified compounds in the sternal glands of male *L. maderae*. 1, Hydroxy-3-butan-2-one; 2, (2*R*, 3*R*)-butanediol; 3, senecioic acid; 4, (*E*)-2-octenoic acid; IS, internal standard

Table 1 Y-maze olfactometer: number of females of *L. maderae* (n=30) attracted to a conspecific adult of either sex, or to male equivalents (ME) of male sternite extract, versus air

Nonresponding Insects (n)	Stimulus A	Number	Stimulus B	Number	P
4	Solvent	13	Air	13	0.578
0	Female	18	Air	12	0.181
0	Male	25	Air	5	<0.001
0	Male	27	Female	3	<0.001
0	1 ME	24	Air	6	<0.001
0	10^{-1} ME	22	Air	8	0.008
3	10^{-2} ME	22	Air	5	<0.001
3	10^{-3} ME	14	Air	13	0.500

lesser quantities: hydroxy-3-butan-2-one (480.2±46.9 ng), (2*R*, 3*R*)-butanediol (51.3± 11.6 ng), senecioic acid (230.8±49.9 ng), and (*E*)-2-octenoic acid (5.3±0.8 ng).

Bioassays The results are shown in Table 1. With the solvent control, about 87% of the females entered the common arm of the olfactometer and distributed randomly between the two choice arms. When the stimulus was a live male or female, all the females entered the olfactometer. Females were not attracted to a female, but were significantly attracted to a conspecific male, compared to the blank choice arm. For most concentrations of male sternite extract tested, females significantly preferred the arm of the olfactometer containing the extract over the blank arm. At concentrations of 10^{-2} to 1 ME, 73 to 81% of females that entered the common branch preferred the arm containing the male extract to the blank arm. There was no significant preference of females to male extract at a concentration of 10^{-3} ME, suggesting that this concentration is around the threshold of the female perception of the male pheromone.

The most volatile component of the male pheromone, hydroxy-3-butan-2-one, was attractive to females when tested at concentrations from 0.1 to 1 ng (Table 2); 69 to 81% of the females that reached the intersection of both choice arms preferred the arm containing hydroxy-3-butan-2-one. Although 83 to 97% of the females reached the common arm when various concentrations of (2*R*, 3*R*)-butanediol were tested, this component was not significantly attractive at any of the concentrations tested (Table 3). Ninety to 93% of the females tested entered the olfactometer when various concentrations of senecioic acid were used (Table 4); however, only the highest amount (7 μg) tested was significantly attractive.

Table 2 Y-maze olfactometer: number of females of *L. maderae* (n=30) attracted to various amounts of hydroxy-3-butan-2-one or air

Nonresponding Insects (n)	Stimulus A	Number	Stimulus B	Number	P
4	0.1 ng	18	Air	8	0.038
3	0.5 ng	22	Air	5	<0.001
1	1 ng	20	Air	9	0.031
5	5 ng	17	Air	8	0.054
1	10 ng	14	Air	15	0.644
3	100 ng	14	Air	13	0.500
2	500 ng	15	Air	13	0.425
6	1 μg	16	Air	8	0.076

Table 3 Y-maze olfactometer: number of females of *L. maderae* (n=30) attracted to various amounts of (2*R*, 3*R*)-butanediol or air

Nonresponding Insects (n)	Stimulus A	Number	Stimulus B	Number	P
2	0.1 ng	17	Air	11	0.172
1	10 ng	16	Air	13	0.068
2	100 ng	15	Air	13	0.425
3	150 ng	17	Air	10	0.124
1	400 ng	16	Air	13	0.356
5	1 μg	13	Air	12	0.500

(*E*)-2-Octenoic acid was not attractive to the females at any of the concentrations tested (Table 5).

When four mixtures of the compounds were tested, only two were significantly attractive (Table 6): the binary mixture of hydroxy-3-butan-2-one/senecioic acid and the quaternary mixture. A number of females entered the common arm of the olfactometer when (2*R*, 3*R*)-butanediol or (*E*)-2-octenoic acid was added to hydroxy-3-butan-2-one. However, when the insects arrived at the intersection of the two arms, the females preferred the air stimulus, suggesting that the mixtures were repellent at a close distance.

Discussion

Cockroaches rely on pheromones for many aspects of reproductive behavior, especially for attraction of a sexual partner over a distance (Roth and Willis, 1954; Barth, 1968). In all the species of cockroaches studied, the emission of a sex pheromone by males or females is associated with a "calling" posture of the emitter (Willis, 1970; Schal and Bell, 1985; Sreng, 1993). The existence of sex pheromones attractive over a distance has been demonstrated in about 16 species (reviewed in Gemeno and Schal, 2004). However, the chemical identity of the attractants has been elucidated only for a limited number belonging to four families: Blattidae (Persoons et al., 1990; Farine et al., 1993, 1994; Nishii et al., 1997), Blattellidae (Nishida et al., 1976, 1979; Schal et al., 1990; Charlton et al., 1993; Nojima et al., 2005), Blaberidae (Sreng, 1990; Sirugue et al., 1992), and Cryptocercidae (Le Quéré et al., 1991). Generally, it is males that are attracted to a sex pheromone emitted by conspecific females (Willis, 1970; Moore and Barth, 1976; Schal, 1982; Schal and Bell, 1985; Abed et al., 1993b,c; Liang and Schal, 1993) but, in some Blattinae (Abed et al.,

Table 4 Y-maze olfactometer: number of females of *L. maderae* (n=30) attracted to various amounts of senecioic acid or air

Nonresponding Females (n)	Stimulus A	Number	Stimulus B	Number	P
2	0.1 ng	13	Air	15	0.425
3	1 ng	12	Air	15	0.351
2	10 ng	13	Air	15	0.425
3	100 ng	14	Air	13	0.500
2	1 μg	14	Air	14	0.575
3	2.8 μg	15	Air	12	0.351
3	7 μg	19	Air	8	0.026

Table 5 Y-maze olfactometer: number of females of *L. maderae* (n=30) attracted to various amounts of (*E*)-2-octenoic acid or air

Nonresponding Insects (n)	Stimulus A	Number	Stimulus B	Number	P
4	0.1 ng	12	Air	14	0.422
2	1 ng	13	Air	15	0.425
5	10 ng	11	Air	14	0.345
4	100 ng	11	Air	15	0.279
1	200 ng	13	Air	16	0.356
12	1 μg	6	Air	12	0.119

1993a), Polyzosteriinae (Farine et al., 1993, 1994), and Oxyhaloinae (Sreng, 1990; Sirugue et al., 1992), it is the male that emits the sex pheromone.

The courtship behavior of *L. maderae* is similar to that of *N. cinerea* (Roth and Barth, 1967). Male *L. maderae* possess a well-developed glandular epithelium lying under the whole area of sternites 2 to 7 (exposed during calling) and tergites 2 to 8 (exposed during the wing-raising posture) (Sreng, 1984). Additionally, the middle part of tergite 2 shows a cuticular excrescence under which several layers of glandular cells are observed (Sreng, 1984). In male *N. cinerea*, the glandular cells are found under sternites 3 to 7 and tergites 2 to 8, whereas the *L. maderae* glandular modification is absent (Sreng, 1984).

In *N. cinerea*, the pheromone is released from the sternal glands. The secretion is a mixture of hydroxy-3-butan-2-one, 2-methylthiazolidine, 4-ethylguaiacol, (2*R*, 3*R*), and (2*R*, 3*S*)-butanediol (Sreng, 1990; Sirugue et al., 1992). Sirugue et al. (1992) showed that all these components are present in lower quantities in the tergal secretion and that 4-ethylguaiacol and 2-methylthiazolidine attract females at very low dosages (0.05 and 0.01 ng, respectively). Quantities of 2-methylthiazolidine and 4-ethylguaiacol increase gradually in males, reach a maximum at 15–20 days, and then decline (Moore et al., 1995). Variation in pheromone quantity with age parallels the age-related attractiveness of males to females, male sexual competence, and male agonistic behavior (Moore and Moore, 1999; Moore et al., 1997, 2001). Hydroxy-3-butan-2-one attracts virgin females at a dosage of 100 ng but appears to repel them at a dosage of 1 μg (Sirugue et al., 1992). Moore et al. (1995) found that the amount of hydroxy-3-butan-2-one increases in males up to age 10–15 days and is then maintained at a relatively constant level, suggesting that this component might have a different role from that of the other compounds.

In *L. maderae*, hydroxy-3-butan-2-one and senecioic acid are the main components of the sex pheromone. Hydroxy-3-butan-2-one is attractive to females when used at low dosages (0.1 to 5 ng), whereas senecioic acid is only attractive at very high dosages (7 μg).

Table 6 Y-maze olfactometer: number of females of *L. maderae* (n=30) attracted to various mixtures containing hydroxy-3-butan-2-one (H3B2), (2*R*, 3*R*)-butanediol [(2, 3)-BUT], senecioic acid (SENE Ac), and (*E*)-2-octenoic acid [(*E*)-2-OCT Ac] or air

Nonresponding Insects (n)	Stimulus A				Stimulus B			P
	H3B2	(2,3)-BUT	SENE Ac	(*E*)-2-OCT Ac	Number		Number	
2	380 ng	160 ng	–	–	11	Air	17	0.172
2	380 ng	–	2.8 μg	–	23	Air	5	<0.001
3	380 ng	–	–	100 ng	10	Air	14	0.124
0	380 ng	160 ng	2.8 μg	100 ng	23	Air	7	0.003

Considering the large amount of the latter compound detected in the sternal glandular epithelium (about 30 μg per male), it is possible that it is emitted in high amounts by a calling male. (2*R*, 3*R*)-Butanediol and (*E*)-2-octenoic acid did not attract females when tested on their own and negated the attractiveness of hydroxy-3-butan-2-one when added to it in binary mixtures.

As noted for *N. cinerea*, the behavioral role of (2,3)-butanediol is unknown. One possibility is that it is a precursor of hydroxy-3-butan-2-one in the sternal glands of both species and is synthesized by partial oxidation of (2,3)-butanediol (Bauer et al., 1990). Alternatively, hydroxy-3-butan-2-one and (2,3)-butanediol could be simultaneously produced by fermentation of glucose by bacteria and fungi (Mac Faddin, 1980; Gottschalk, 1986).

In laboratory conditions, sexually excited cockroach males may court females of other species, but successful interspecific mating never occurs. Interspecific attraction of males by female sex pheromone has been reported only in the genera *Periplaneta* and *Blatta* (Simon and Barth, 1977). Structural similarity of the sex pheromones (periplanones) among *Periplaneta* species and *Blatta orientalis* has been demonstrated using bioassays of synthetic and isolated pheromones (Seelinger, 1985; Takahashi et al., 1988, 1995; Takegawa and Takahashi, 1989; Nishii et al., 1997). Colonies of *L. maderae* and *N. cinerea* can be kept together without any interspecific mating (Everaerts et al., 1997). Olfactometer tests have shown that females of either species are not attracted to males of the other (Sirugue, 1992). Hydroxy-3-butan-2-one may play a role in this lack of cross attraction. This chemical is attractive to *L. maderae* at low dosages (0.1 to 5 ng) and attractive to *N. cinerea* at higher dosages (100 ng) (Sirugue et al., 1992). Other chemicals could contribute to the specificity of the respective pheromones when the female is closer to the male (e.g., senecioic acid for *L. maderae*, 2-methylthiazolidine and 4-ethylguaiacol for *N. cinerea*).

During courtship, males of both *L. maderae* and *N. cinerea* display two successive postures: the first, a calling posture when the sex pheromone is released, and the second, after antennal contact with a conspecific female, a wing-raising posture in which the tergal glands are exposed (Roth and Willis, 1954; Sreng, 1993). The tergal secretions are complex mixtures usually considered as "aphrodisiacs" (Brossut et al., 1975). As found in other species of cockroaches, there are exocrine proteins in the cuticular waxes (Abed et al., 1995; Korchi et al., 1998), but little is known about their function (Cornette et al., 2001, 2002, 2003). In both species, the identified volatile components of the tergal glands are the same as those found in the sternal gland. These volatile chemicals could enhance the effect of the aphrodisiac secretions, eliciting the female to climb on to the male's back.

Aggressive behavior, dominant–subordinate relationships, hierarchy, and territoriality in males are well-known behaviors in various species of cockroaches (Ewing, 1972, 1973; Simon and Barth, 1977). All these behaviors have been extensively studied in male *N. cinerea* (Ewing, 1972; Ewing and Ewing, 1973; Bell et al., 1979; Breed et al., 1980; Sirugue et al., 1992). Male dominance hierarchies and territoriality are mediated by chemical signals, and these signals are also involved in female mate choice (Smith and Breed, 1982; Schal and Bell, 1983; Moore, 1989, 1990; Sreng, 1990; Moore et al., 1995; Everaerts et al., 1997). Moore et al. (1995) demonstrated that female *N. cinerea* preferred dominant males to subordinate ones and further showed that the sternal gland of dominant males contained less sex pheromone than that of subordinate males. They hypothesized that dominant males "bully" subordinates to prevent them from releasing pheromone and having access to females. Additionally, Moore and Moore (1999) showed that female choice is

based solely on the amount of hydroxy-3-butan-2-one. As stated previously, *N. cinerea* and *L. maderae* belong to the small subfamily Oxyhaloinae, and aggressive behavior between males exists in both species (Bell et al., 1979). It would be interesting to determine if hydroxy-3-butan-2-one is multifunctional in *L. maderae*, mediating pheromonal attraction, dominant/subordinate relationships between males, and mate choice by females.

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J Chem Ecol (2007) 33:417–434
DOI 10.1007/s10886-006-9230-9

Chemical Identification of MHC-influenced Volatile Compounds in Mouse Urine. I: Quantitative Proportions of Major Chemosignals

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Received: 24 August 2006 / Revised: 24 August 2006 / Accepted: 20 November 2006 /
Published online: 27 December 2006
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Abstract The genes of the major histocompatibility complex (MHC) are highly polymorphic loci that encode cell surface proteins, class I and II molecules. They present peptide antigens to T cells and thereby control immunological self/nonself recognition. Increasing evidence indicates that MHC genes also influence odor and mating preferences; however, it is unclear how. Here we report the results of chemical analyses of male mouse urinary odors collected from a variety of mouse strains, including MHC-congenics, recombinants, mutants, and transgenics (i.e., β2 microglobulin "knockouts," which lack class I expression, and transporters associated with antigen processing (TAP) knock-outs). After the identification of volatile odor components by gas chromatography/mass spectrometry, the odor profiles of urine samples were analyzed quantitatively by using stir bar sorptive extraction and gas chromatography/atomic emission detection. Results showed that MHC genes influenced the amounts of testosterone-mediated pheromones, sulfur-containing compounds, and several carbonyl metabolites. This is the first report to quantitatively link known mouse pheromones to classical, antigen-binding MHC loci. Surprisingly, these compounds were not influenced by TAP genes, even though these loci are MHC-linked and play a role in peptide presentation. Whereas identification of MHC-determined odorants does not reveal their metabolic origin, some constituents were also present in blood serum, and their levels were not altered by antibiotics.

Keywords Individual odor · Volatile pheromones · Gas chromatography/mass spectrometry · Gas chromatography/atomic emission detection · MHC genes · Mouse urine

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Introduction

Since the discovery that odor and mating preferences are influenced by the genes of the major histocompatibility complex (MHC or H-2 in mice) in house mice (*Mus musculus domesticus*) (Yamazaki et al., 1976), several additional laboratories have found evidence for these effects (reviewed in Penn and Potts, 1998a, 1999; Penn, 2002; Beauchamp and Yamazaki, 2003). Using a Y-maze olfactometer, it was found that trained mice can discriminate among the odors of urinary samples from mice that differ genetically only in the MHC region (MHC-congenic strains) (Yamaguchi et al., 1981; Yamazaki et al., 1983; Penn and Potts, 1998b). In more recent experiments, untrained mice were able to distinguish among the odors of mouse strains that differed genetically only at a single MHC locus (Penn and Potts, 1998b; Carroll et al., 2002). Several studies now suggest that the MHC-dependent mating preferences are not confined to mice because MHC effects have been implicated in mating preferences of fish, lizards, birds, and even humans (reviewed in Penn, 2002). It has been postulated that this mating preference functions to increase the resistance of an individual's offspring to infectious diseases, either by increasing (or optimizing) MHC-heterozygosity or by providing a "moving target" against rapidly evolving pathogens (Penn and Potts, 1999). Another possibility is that MHC similarity (used to recognize kin) and disassortative mating preferences function to avoid inbreeding (Brown and Eklund, 1994). Indeed, female mice are more likely to retrieve MHC-similar than dissimilar pups, which supports the kin-recognition hypothesis (Yamazaki et al., 2000). A great deal of evidence indicates that MHC genes influence urinary odor, and a number of mechanisms have been suggested to explain how this occurs (Penn and Potts, 1998a; Beauchamp and Yamazaki 2003). Using state-of-the-art analytical methodologies, the goal of our studies has been to identify and quantitatively measure the major volatile components of urine that are influenced by MHC genes.

As an extension of our previous work, we examined urinary odors of male house mice (*M. musculus domesticus*). Mice use urine to scent-mark, and their urine is a rich source of biological signals, including primer and releaser pheromones (reviewed in Novotny et al., 1999a; Novotny, 2003). Urine confers information such as gender and endocrine status, which is perceived through the main and accessory olfactory senses (Leinders-Zufall et al., 2000; Sam et al., 2001). Mouse pheromones are relatively small and hydrophobic molecules, and MHC-mediated odorants may also fall into the same category. A number of studies indicate that the MHC-dependent odors are volatile compounds (reviewed in Penn and Potts, 1998a). MHC-dependent odorants may be either (a) direct degradation products of MHC molecules and/or peptides; (b) secondary metabolites of biochemical pathways moderated by the MHC; (c) the result of MHC-specific communities of microbial flora (the so-called microflora hypothesis; Howard, 1977; Schellinck et al., 1991); or (d) some combination of these mechanisms, e.g., metabolic products from microflora degradation of MHC-molecules or peptides (Penn and Potts, 1998a).

The physiological fluids of mammals contain complex mixtures of structurally varied metabolites, and their volatile constituents can be analyzed by combined capillary gas chromatography/mass spectrometry (GC/MS). This approach was used in the first studies characterizing the urinary constituents of mice from different strains (Novotny et al., 1980; Schwende et al., 1984, 1986). These studies established that (a) mouse urine contains a number of structurally unique substances; (b) substantial quantitative differences in the volatile substance profiles can be observed among strains; and (c) if any quantitative differences are caused by MHC, they should be relatively minor in comparison to differences influenced by non-MHC (background) genes.

The first bioanalytical study to statistically evaluate the volatiles' profiles of congenic mice found a correlation between the MHC genetic relatedness and chromatographic profiles of urine (Schwende et al., 1984). It seemed that a pattern, or a ratio of several components (ubiquitous urinary metabolites), rather than single components were primarily responsible for the MHC discrimination. A later study (Eggert et al., 1996) also used GC profiling to distinguish more general (multiple gene controlled) strain- and more narrowly controlled MHC-related differences. Singer and coworkers (1997) examined acidic metabolites, arguing that the acidic products of metabolism are far more common than neutral ones and thus more likely to be involved in the MHC signaling. Whereas their profiles of acids in male mouse urine contained 32 major constituents, only seven substances were identified, with one (phenylacetic acid) deemed important. Furthermore, they only examined MHC-congenic strains, which differ at many genes in the MHC region and, therefore, it is unclear whether these acidic compounds were influenced by MHC class I and II antigen presenting loci. In a radically different technical approach, it was shown that a set of semiconductor sensors (the so-called "electronic nose") could distinguish between the odors (from urine and blood serum) of different MHC congenic mouse strains and mutants (Montag et al., 2001). Headspace analysis by GC/MS in the same study identified only a limited number of compounds, and was not supportive of the importance of organic acids. Recently, Willse and coworkers (2005) used two-dimensional GC with MS detection and chemometric analyses to investigate differences between two congenic strains (carrying *b* vs. *k* haplotypes on C57BL/6J background) and suggested that approximately 80 compounds may differ (with 23 compounds being identified). Among these identified compounds, there were two previously known volatile chemosignaling compounds with proven pheromonal activities (Novotny et al., 1999a,b; Novotny, 2003).

Whereas no investigation to date has identified an allele- or haplotype-specific compound or a set of such substances (i.e., those that would be present in one haplotype and absent in another), some previous investigations allude to the possibility of specific ratios or "patterns" of volatiles. To examine the hypothesis that variations in odor are mainly of a quantitative nature (Schwende et al., 1984), it is necessary to measure the odor profiles with sufficient precision to detect small but biologically significant differences in concentrations. Our results here provide such quantitative data for a group of known mouse chemosignaling compounds. For the study, we used an element-specific coupled GC–atomic emission detector system (GC-AED) (carbon-, sulfur-, and nitrogen-specific lines) to obtain quantitative profiles of urinary volatiles. Additionally, AED provided the necessary precision for the minute quantities of sulfur compounds, without interference from other urinary constituents. Nitrogen line signals also supported the previous identifications of the nitrogen-containing compounds by GC-MS.

Methods and Materials

Experimental Animals Male mice of seven different strains with B6 and B10 backgrounds were purchased at 5–6 weeks of age (Jackson Laboratory, JAX Research Systems, Bar Harbor, ME, USA). To identify the odors that are influenced by MHC genes and control effects from background genes, we used MHC congenic mouse strains because the only consistent genetic differences among these strains are in the MHC region. For example, strain C57BL/10J (B10 background) carries the *b* haplotype (H-2^b), whereas its congenic strain, B10.D1-H2<q>, carries the *q* haplotype (H-2^q). We also used recombinant, mutant,

and knock-out transgenic strains to examine effects from classical MHC loci. The mouse strains used in this study are summarized in Table 1. One of the B6 strains was a mutant bm1 (B6.bm1) with a small spontaneous mutation in two amino acids in the peptide-binding region encoded by a single class I locus (Carroll et al., 2002). One of the B10 strains (B10.A) was a recombinant strain differing from B10.BR by one class I locus. Additionally, two congenic BALB strains with *b* and *k* haplotypes were included to compare strains of different background with the same MHC haplotype. Finally, two mutant strains of C57BL/6J were added to compare the animals that lack MHC class I expression or the assembly of MHC class I molecules (β2 microglobulin "knockouts"). The mice were housed individually in polypropylene cages with opaque sides and fitted wire lids (cage size: 29×18×13 cm). Wood-shaving bedding (Sani-chips, P.J. Murphy Forest Products, Montville, NJ, USA) was changed once a week. Food (Lab Diet for mice, PWI Nutrition International, Brentwood, MO, USA) and water were provided *ad libitum*. The animal room was maintained at 21–22°C, 70% relative humidity, and 12-hr light/12-hr dark lighting regime, with lights on at 0600 hours.

Urine Collection Urine samples were collected at 9–10 weeks of age. Mice were placed in a polycarbonate cylindrical urine-collecting apparatus (22 cm diameter×19 cm height) from 0900 hours for 2–4 hr. The urine collection apparatus has partitions that divide the area equally into five sectors to collect urine from five different mice simultaneously without mixing the samples. The bottom of the urine-collecting apparatus was made of a wire mesh. A plastic strip was laid beneath the urine collection apparatus, and the strip was checked every 5 min, while urine (total of at least 2 ml volume) was collected using a Pasteur pipet. The collected urine was placed in an Eppendorf tube, kept frozen using dry ice, and subsequently kept at −70°C until it was chemically analyzed. The strip was

Table 1 Mouse strains used in the study

Name of strain	Abbreviation	Class I[a] H-2k	Class II[a] I-A	Class II[a] I-E	Class I[a] H-2D	*n*
B6 background						
C57BL/6J (host)	B6.6J	b	b	bEa0	b	6
B6.C-H2<bm1>/ByJ (minor mutation)	B6.bm1	bm1	b	bEa0	b	6
B6.AK-H2<k>/FlaEg	B6.AK	k	k	k	k	6
B10 background						
C57BL/10J (host)	B10.10J	b	b	bEa0	b	8
B10.BR-H2<k>H2-T18<a>/SgSnJ	B10.BR	k	k	k	k	8
B10.A-H2<a>H2-T18<a>/SgSnJ	B10.A	k	k	k	d	8
B10.D1-H2<q>/SgJ	B10.D1	q	q	qEa0	q	8
BALB background						
C.C3-H2<k>/LilMcJ	BALB/k	k	k	k	k	6
C.10-H2<b>/LilMcdJ	BALB/b	b	b	bEa0	b	5
B6 knockout mutants						
B6.129S2-*Tap1*^{tm1Arp}/J	B6.Tap1[b]	–	b	b	–	6
B6.129P2-*B2m*^{tm1Unc}/J	B6.B2m[c]	–	b	b	–	6

[a] MHC gene-coded glycoproteins that present peptides to T lymphocytes on cell surfaces

[b] B6.*Tap1* mice are mutants of B6.6J strain. They are defective in the stable assembly and intracellular transport of class I molecules, showing severely reduced levels of class I surface molecules

[c] B6.*B2m* mice are mutants of B6.6J strain. They have little if any MHC class I protein expression on the cell surface

replaced whenever a sufficient urine volume (2 ml) was collected to avoid stale urine and contamination of samples from adjacent collection sectors.

Antibiotic Treatment After the initial collection of samples was completed, the effect of antibiotic treatment on the urinary volatile profiles was investigated (two additional sample collections). After the first collection of urine, half of the animals went through antibiotic treatment (experimental group). The other half served as control mice and went through the collection of urine again at the same time as the experimental group. For the experimental group, tetracycline was added to the drinking water of each mouse (1.3 g/l). Sugar was added to the tetracycline-treated water (2.6 g/l) to mask the bitter taste. The control group received sugar water only. Urine was collected from mice (and controls) 1 week after they started to receive tetracycline-treated water. Urinary volatile constituents were compared before and after the antibiotic treatment.

Blood Collection After all urine collections were completed and after a further 4 weeks for antibiotic clearance, the animals were anesthetized using tribromoethanol. Blood was collected directly from the heart using a 25G 1/2-in needle and a 1-ml syringe. The blood collected was centrifuged at ca. 2,000 rpm for 20 min, and the serum was removed and frozen on dry ice. The frozen serum was kept at −70°C until it was analyzed. The experimental procedure and housing conditions were approved by the Bloomington Institutional Animal Care and Use Committee of Indiana University.

Reagents and Analytical Methods All compound identifications were verified through authentic standards purchased from Aldrich (Milwaukee, WI, USA) or synthesized in our laboratory according to the previously described synthetic methods (Wiesler et al., 1984; North and Pattenden, 1990; Novotny et al., 1999b). Stir bars (Twister™, 10 mm, 0.5 mm film thickness, 24 μl polydimethylsiloxane volume) used for sorptive extraction were purchased from Gerstel GmbH (Mülheim an der Ruhr, Germany). Volatile and semivolatile compounds were extracted from 0.5 ml of urine in 20-ml capped glass vials for 60 min with a Twister™ stir bar. Urine samples were first diluted with 1.5 ml water (high-purity OmniSolv® water, EM Science, Gibbstown, NJ, USA). As an internal standard, 16 ng of 7-tridecanone in 20 μl of ethanol (Aldrich) was added to each vial. Stirring speed was 800+ rpm on the Variomag Multipoint HP 15 stirplate (H+P Labortechnic, Oberschleissheim, Germany). Prior to extraction, all glassware was rinsed with acetone and dried at 80°C. After extraction, the stir bars were rinsed with a small amount of distilled water, dried gently on a paper tissue, and placed in the thermal desorption autosampler (TDSA) tube (a product of Gerstel) for GC or GC-MS quantitative analysis. GC equipment for quantitative analysis consisted of an Agilent GC Model 6890 with an AED (Model G2350A, Agilent Technologies, Wilmington, DE, USA) and a TDSA (Gerstel). The GC was fitted with an HP-5MS column (30 m×0.25 mm, ID, 0.25 μm film thickness, Agilent). Samples were thermally desorbed with a TDSA automated system, directly into cooled injection assembly (CIS-4) of the GC, operated in splitless mode. The temperature program for desorption was 20°C (0.5 min), then 60°C/min to 280°C (10 min). Temperature of the transfer line was set at 280°C. The CIS was cooled with liquid nitrogen to −60°C. After desorption and cryotrapping, the CIS was heated at 12°C/sec to 280°C, with a hold time of 10 min. The CIS inlet was operated in the solvent vent mode, with a vent pressure of 14 psi, a vent flow of 30 ml/min, and a purge flow of 50 ml/min. The GC oven was programmed from 40°C for 5 min, then 2°C/min, to 200°C hold

10 min. The carrier gas head pressure was 14 psi (flow rate, 1.2 ml/min). The GC was operated in constant flow mode. The emission lines for carbon (193 nm), sulfur (181 nm), and nitrogen (174 nm) were monitored with the AED detector.

Compounds were identified with an Agilent 6890N GC connected to 5973i MSD MS (Agilent Technologies). The GC column and temperature program were identical to those used with the GC-AED. Electron input ionization mode was used with a scan rate of 2.4 scans/sec over the mass range of 41–350 amu. The transfer line temperature was 280°C and the ion source and quadrupole temperatures were 230 and 150°C, respectively. The TDSA–CIS sample introduction setting was identical to that described above with the GC-AED system.

Volatile compounds in serum were extracted by the stir bar method after digesting proteins with a nonspecific protease enzyme. Serum (200 μl) was placed in a clean 25-ml sample vial and diluted with 10 ml HPLC grade water and 10 ml 50 mM ammonium bicarbonate buffer, pH 8.3. Then, 2 mg of protease (Sigma protease type XIV from *Streptomyces griseus*, 5.2 units/mg) was added to the vial. Enzyme digestion was carried out for 18 hr in foil-wrapped vials (protected from light) at room temperature. After digestion, 20 μl of the internal standard solution in ethanol, containing 16 ng of 7-tridecanone, was added. Stir bar extraction was performed for 2 hr, and the sample was introduced into the GC-MS system as described for the urine analysis. Digestion of serum proteins was deemed necessary for the extraction of small volatile compounds due to possible competitive binding of compounds of interest on the surfaces of intact proteins.

Quantitative Evaluations and Statistical Analyses As the basis for quantitative comparisons of urinary chromatographic profiles, the peak areas (GC-AED) were integrated for each identified component and statistically evaluated for different mouse groups. Typically, reproducibility for the compound peak area measurements in mouse urine was ±5.0% (relative standard deviation, N=4) (see also Soini et al., 2005). Comparisons to the peak areas obtained with authentic compounds at known concentrations (ng/ml) or the internal standard peak area (16 ng of 7-tridecanone) were used to estimate the actual concentration ranges. The AED detector operated well within the linear range at the submicrogram levels for the detected compounds. Typically, the measured correlation coefficients (R^2) for the analytes were >0.99.

The peak area of the internal standard was monitored as a quality control measure for reproducibility of extraction, and chromatography and was not used for statistical evaluations. ANOVA was employed for statistical comparisons, and Tukey's test was used for *post hoc* comparisons.

Results

Comparison Among Mice With the Same Background and Different MHC Through sampling two different host strains, C57BL/6J (B6.6J) and C57BL/10J (B10.10J) (Table 1), and several MHC haplotypes and mutants within these strains, we were able to quantitatively compare the profiles of mouse urinary volatiles and assess the differences in major constituents that may occur due to a variation in the MHC region. Examples of our results from compound profiling comparisons are shown in Figs. 1 and 2. The volatiles profiles were independently assessed by GC-MS (data not shown) to verify the identities of previously known chemosignals (Schwende et al., 1986; Novotny et al., 1999a), in addition to identifying some new compounds. Statistical analyses carried out for 18 major urinary components showed significant differences (marker compounds) among the different strains for most compounds (see Table 2).

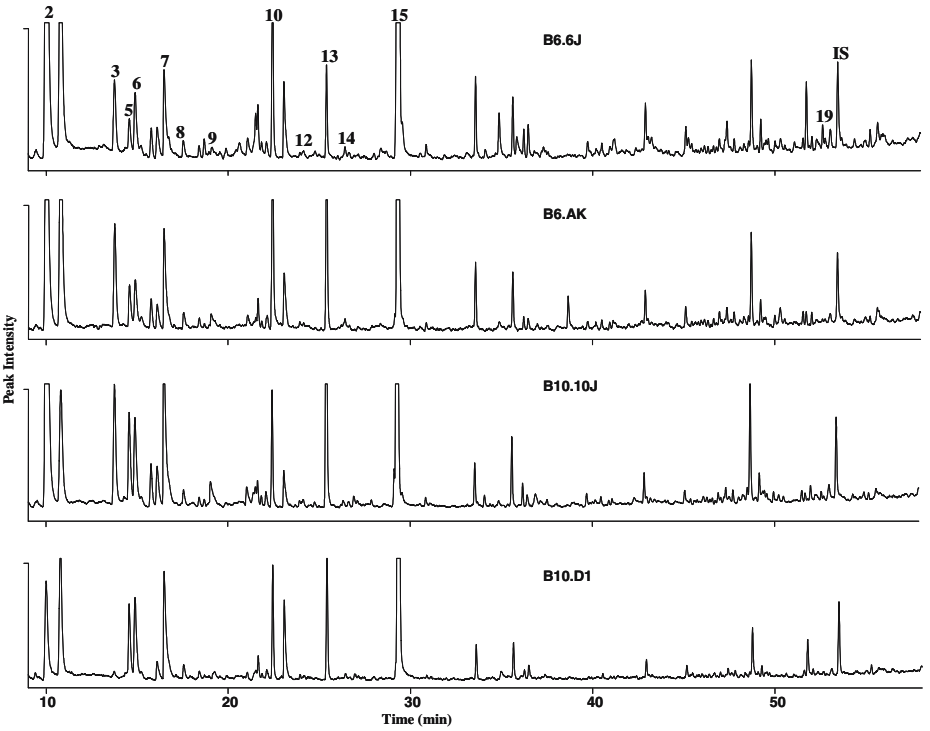


Fig. 1 A comparison of male mouse urinary profiles measured by GC-AED, using the carbon line at 193 nm, in different strains and haplotypes. Analytical conditions are described in the text. *Numbers* refer to compounds: *2*, *3*, *7*, DHF; *5*, 2-heptanone; *6*, 5-hepten-2-one; *8*, 2-ethyl-5-methyl-5,6-dihydro-(4H)pyran; *9*, 6-methyl-6-hepten-3-one; *10*, 6-methyl-5-hepten-3-one; *12*, limonene; *13*, DHB; *14*, acetophenone; and *19*, β-farnesene. *IS* internal standard

Among the marker compounds, highest concentrations were detected for dihydrofurans (DHF) (500–2,000 ng/ml), dehydro-*exo*-brevicomin (DHB) (100–400 ng/ml), and 2-*sec*-butyl-4,5-dihydrothiazole (SBT) (150–900 ng/ml). Two carbonyl compounds were found at medium concentration levels (2-heptanone: 7–150 ng/ml, 5-hepten-2-one: 12–160 ng/ml), whereas 3-hepten-2-one levels were somewhat lower (2–5 ng/ml). Sulfur compound levels (dimethyl disulfide and methyl (methylthio)methyl disulfide) were 10–40 ng/ml and 15–30 ng/ml, respectively. Another sulfur compound, bis(methylthio)methane, was found at even lower levels (1–3 ng/ml). Additional GC-MS analyses showed β-farnesene at relatively low levels (2–5 ng/ml) to be a statistically significant marker compound as well. Chemical structures of all identified components are summarized in Fig. 3.

We found significant differences in the excretion of certain components even for such a subtle genetic difference as a few amino acids in the peptide binding region of a single class I gene (B6.bm1 mutants). This is in agreement with our previous results (Schwende et al., 1984) that the differences in female mouse urinary profiles between the background strains can be greater than those caused by different MHC haplotypes among the host strain and its mutant strains. Eight representative examples of this distinction are shown in Fig. 4. For example, the measured peak areas of SBT were significantly different between B6.6J strain and its congenic strain B6.AK in SBT (Fig. 4h, Table 2). B6.bm1 differed from B6.AK in dimethyl disulfide and bis(methylthio) methane (Fig. 4b, c, Table 2). The three congenic

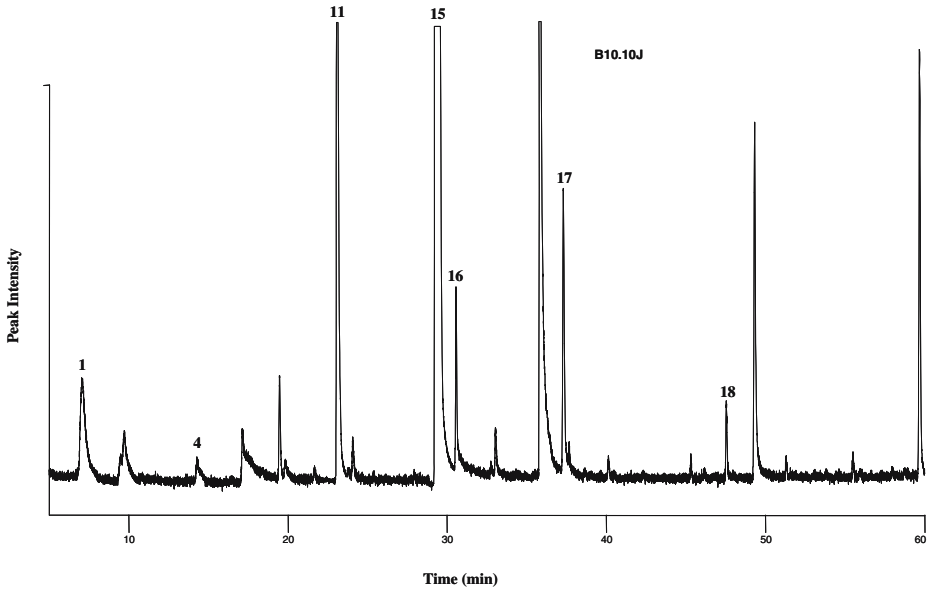


Fig. 2 A selective sulfur line measurement (181 nm) of the male mouse urinary profile by GC-AED (B10.10J strain). *Numbers* refer to compounds: *1*, dimethyl disulfide; *4*, bis(methylthio)methane; *11*, IPT; *15*, SBT; *16*, methyl (methylthio)methyl disulfide; *17*, benzothiazole; and *18*, *N*-(methylthio)methylaniline (tentative identification)

strains of B10.10J showed significantly smaller peak areas for six (B10.A, B10.BR) or seven compounds (B10.D1). The compounds that showed significantly smaller peak areas were dimethyldisulfide (in all of the three congenics of B10.10J), DHF (in B10.D1), bis (methylthio)methane (in all three congenics of B10.10J), 2-heptanone (in all three congenics of B10.10J), 6-methyl-5-hepten-3-one (in B10.A and B10.D1), isopropyl-4,5-dihydrothiazole (IPT) (B10.A and B10.BR), DHB (B10.D1 and B10.BR), and SBT (in all three congenics of B10.10J) (Fig. 4, Table 2). We also found considerable variations within each strain.

From all evaluated components, significant differences in peak areas for eight urinary components (dimethyl disulfide, DHF, bis(methylthio)methane, 2-heptanone, 6-methyl-5-hepten-3-one, IPT, DHB, and SBT) and a lack of significant differences for five other urinary components (5-hepten-2-one, 2-ethyl-5-methyl-5,6-dihydro-(4H)-pyran, methyl (methylthio)methyl disulfide, benzothiazole, and tentatively identified *N*-(methylthio) methylaniline) suggest that the levels of the former eight components reflect variations at the MHC. Figure 4 also shows the differences in peak areas according to the haplotype in the MHC region. Surprisingly, the amounts of 2-heptanone, dimethyl disulfide, and bis (methylthio)methane were larger in the B6.bm1 mutant (bm1, b, b, b) compared to the other genotypes (Fig. 4a–c). The amounts of DHB, 6-methyl-5-hepten-3-one, IPT, and DHF were larger in the haplotype *b* (b, b, b, b), especially in B6.6J, compared to the other haplotypes (Fig. 4d–g). However, even with the same haplotype *b*, there were strain differences, which suggest that regions other than MHC loci may have a role in determining the excretion of these compounds (Fig. 4d–g). When comparing SBT, the averages of the *b* and bm1 mutants were almost the same, but statistically significant differences were seen between haplotypes *b*, again especially in B6.6J and *k*, *k*+*d* (recombinant) and *q* (Fig. 4h).

Table 2 Results of ANOVA on the average peak areas (arbitrary units ± SD) for putative chemosignals

Compound	Number[a]	*F* ratio	*P*	*Post hoc* test, $P<0.05$
Dimethyl disulfide	1	$F_{6,43}=4.01$	0.003	B6.bm1>B6.AK, B10.A, B10.D1, B10.BR
DHF[b]	2,3,7[c]	$F_{6,49}=2.93$	0.016	B6.6J>B10.D1
Bis(methylthio)methane	4	$F_{6,42}=3.80$	0.004	B6.bm1>B6.AK, B10.10J, B10.A, B10.D1, B10.BR
2-Heptanone	5	$F_{6,43}=5.49$	<0.001	B6.bm1>B10.10J, B10.A, B10.D1, B10.BR
5-Hepten-2-one	6	$F_{6,43}=1.78$	NS	
2-Ethyl-5-methyl-5,6-dihydro-(4H)pyran	8	$F_{6,40}=0.92$	NS	
6-Methyl-6-hepten-3-one	9	#		
6-Methyl-5-hepten-3-one	10	$F_{6,35}=3.38$	0.01	B6.6J>B10.A, B10.D1
IPT	11	$F_{6,43}=3.89$	0.003	B6.6J>B10.A, B10.BR
Limonene	12	#		
DHB	13	$F_{6,43}=2.80$	0.022	B6.6J>B10.D1, B10.BR
Acetophenone	14	#		
SBT	15	$F_{6,43}=4.48$	0.001	B6.6J>B6.AK, B10.A, B10.D1, B10.BR
N-(methylthio)methylaniline (tentative)	16	$F_{6,43}=2.05$	NS	
Benzothiazole	17	$F_{6,38}=0.91$	NS	
Methyl (thiomethyl)methyl disulfide	18	$F_{6,35}=0.55$	NS	

Sharp signs mean that ANOVA was not conducted because of the small number of data points

NS = not significant

[a] Shown in chromatograms in Figs. 1 and 2

[b] Dehydration products of 6-hydroxy-6-methyl-3-heptanone

[c] Peak areas of three structurally related DHF were summed in the ANOVA analyses

Comparison Among Mice With the Same MHC and Different Background Mouse strains with the same MHC haplotypes, but with large differences in the gene characteristics in other regions, were chosen to confirm that the differences in the amounts of compounds seen in the two host strains with the same MHC haplotype *b*, or the differences in the amounts of compounds seen in the two congenic strains of these host strains with the same MHC haplotype *k*, will become larger when the gene differences in the regions other than MHC are also increased. A congenic mouse strain of BALB, BALB/b, was chosen to compare with B6.6J and B10.10J because it has the same haplotype *b* in the MHC. Another congenic strain derived from the host BALB, BALB/k, was chosen to compare with B6.AK and B10.BR, which are congenic strains of B6.6J and B10.10J, with haplotype *k* in the MHC region.

The average amounts of three compounds were significantly different for BALB/b compared with B6.6J, and the total sum of peak areas was also significantly different (Table 3). Once again, the compounds that showed differences were DHF, 5-hepten-2-one, and SBT (Table 3, Fig. 4f, h) and all of them were smaller in BALB/b. There were no significant differences between BALB/b and B10.10J and between B6.6J and B10.10J. Even with the same MHC genotype, the amount of each compound thus varies, depending on the differences in the genotype in other genetic regions. When the differences in other regions become larger, the amount of compounds tends to increase, also.

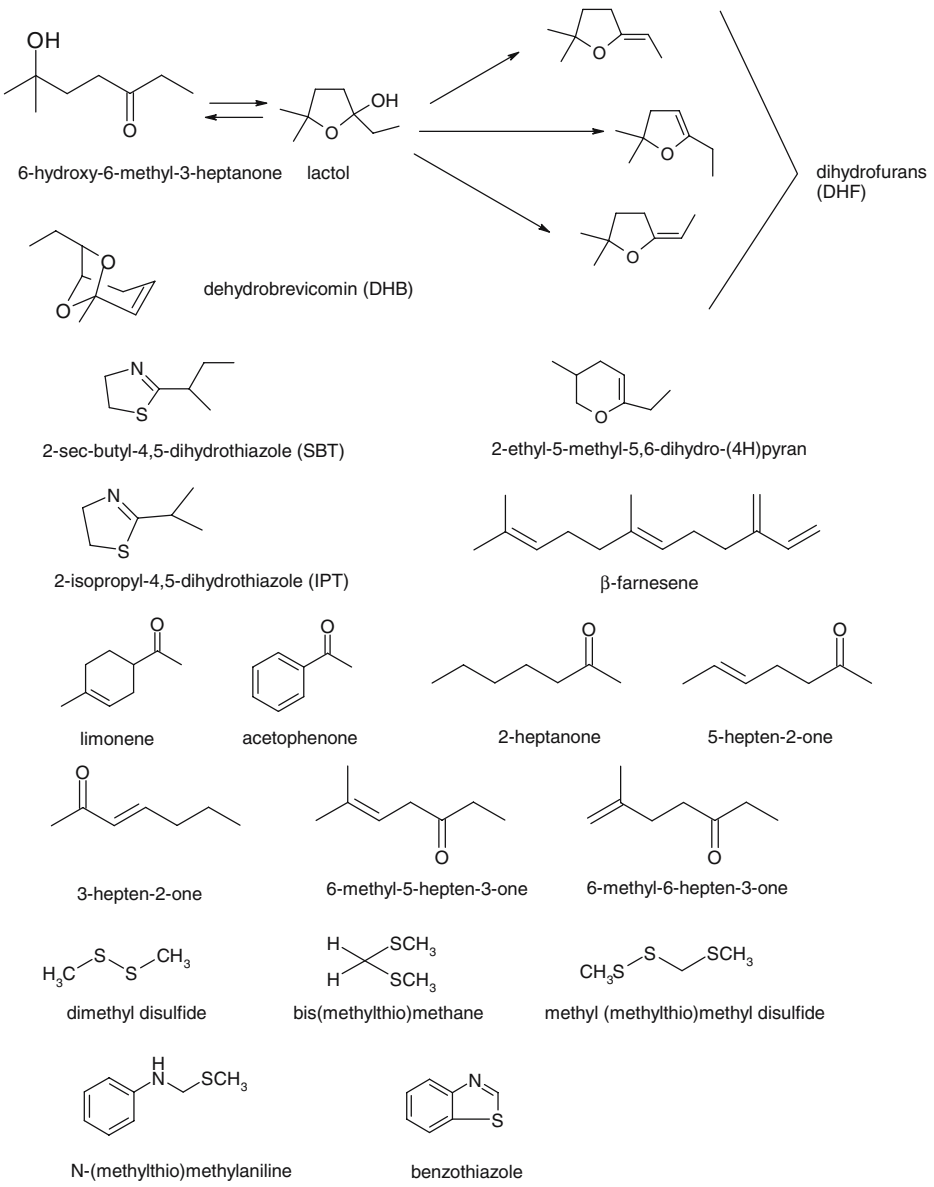


Fig. 3 Chemical structures of the identified compounds

Similar differences were found in a comparison among BALB/k, B6.AK, and B10.BR, which have the same haplotype *k* in the MHC region. There were five compounds that showed differences in peak areas (Table 3, Fig. 4a, d, e, h for compounds other than 5-hepten-2-one) with regard to a strain. *Post hoc* tests revealed that there were no differences between the B6.AK and B10.BR strains. BALB/k produced more DHB than the other two strains (Fig. 4d), but for the other four compounds, the amounts were significantly smaller in BALB/k when compared to B6.AK (2-heptanone (Fig. 4a) and 5-hepten-2-one) and B10.BR (6-methyl-5-hepten-3-one and SBT (Fig. 4e, h)). These results confirm that, even

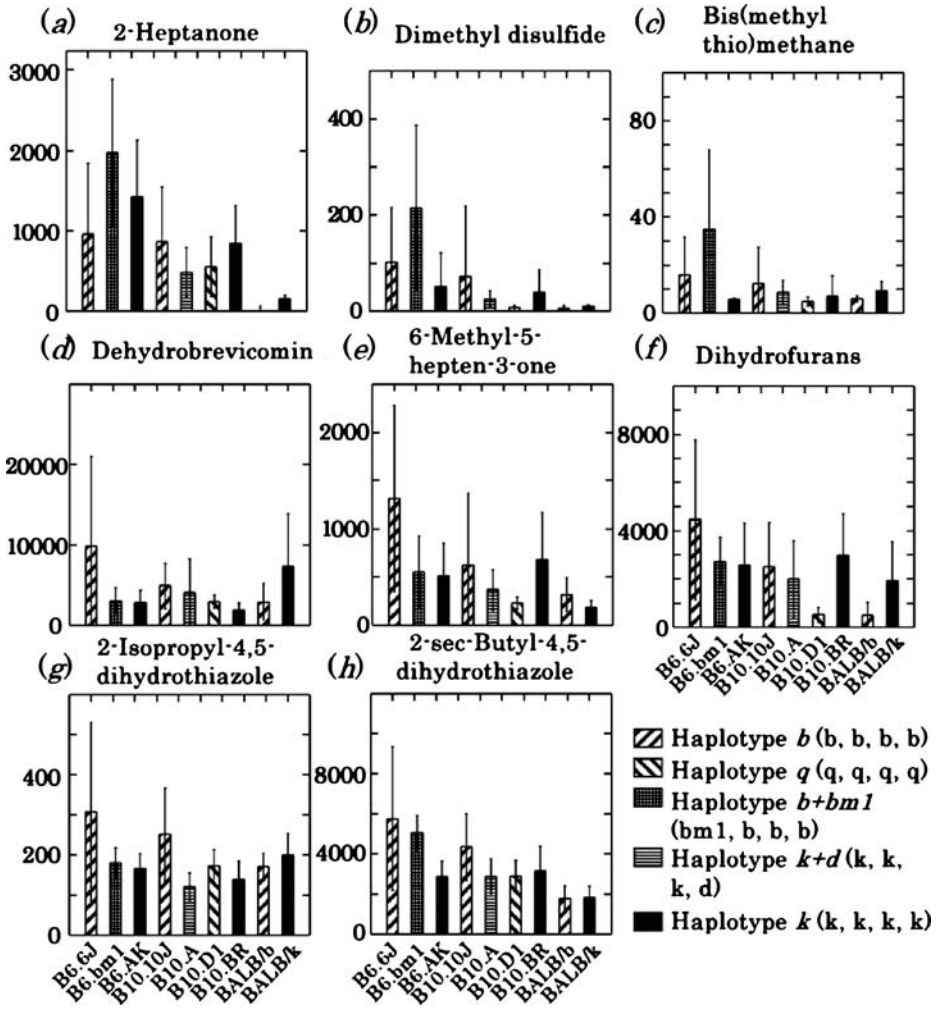


Fig. 4 Comparison of the average peak areas (±SD) according to strains and MHC haplotypes. **a** 2-heptanone, **b** dimethyl disulfide, **c** bis(methylthio)methane, **d** DHB, **e** 6-methyl-5-hepten-3-one, **f** DHF combined, **g** IPT, and **h** SBT

with the same genotype in the MHC region, these compounds vary quantitatively depending on differences in background genes, and when the differences outside the MHC gene regions become larger, the differences in urinary volatiles also increases.

Significant differences in the amounts of urinary volatiles were seen between the congenics of BALB, the B6 and B10 strains, indicating that the differences in urinary chemical profiles increased with increasing differences in background genes. Such a tendency was also seen when the relative percentages of the peak areas of all 16 measured compounds were compared. In Fig. 5, we show the relative percentages of the peak sizes for compounds in the three strains that have the same *b* haplotypes (Fig. 5a) and the three strains that have the same *k* haplotypes (Fig. 5b) in the MHC region. The two C57BL strains (B6 and B10) had quite similar relative percentage profiles when compared to the

Table 3 Average peak areas (arbitrary units ± SD) for the individual compounds in BALB/band BALB/k

Compound	Haplotype b: BALB/b					Haplotype k: BALB/k				
	Mean	±SD	*F* ratio	*P*		Mean	±SD	*F* ratio	*P*	
Dimethyl disulfide	7	4	$F_{2,16}$=1.15	NS		11	3	$F_{2,16}$=1.167	NS	
DHF	5,005	4,716	$F_{2,16}$=5.19	*P*=0.018	a	19,098	14,865	$F_{2,17}$=0.824	NS	
Bis(methylthio)methane	6	1	$F_{2,14}$=0.600	NS		9	3	$F_{2,16}$=0.608	NS	
2-Heptanone			$F_{1,12}$=0.06	NS		159	32	$F_{2,15}$=8.702	*P*=0.003	b
5-Hepten-2-one	274	110	$F_{2,16}$=4.59	*P*=0.027	a	381	146	$F_{2,17}$=4.111	*P*=0.035	b
2-Ethyl-5-methyl-5,6-dihydro-(4H)pyran	133	41	$F_{2,14}$=2.47	NS		233	117	$F_{2,15}$=3.365	NS	
6-Methyl-6-hepten-3-one			#			150		#		
6-Methyl-5-hepten-3-one	319	151	$F_{2,11}$=3.29	NS		187	66	$F_{2,16}$=3.683	*P*=0.048	c
IPT	170	30	$F_{2,16}$=1.43	NS		199	49	$F_{2,17}$=3.423	NS	
Limonene			#					#		
DHB	2,935	1,963	$F_{2,16}$=2.06	NS		7,303	5,926	$F_{2,17}$=4.827	*P*=0.022	d
Acetophenone			#					#		
SBT	1,779	554	$F_{2,16}$=5.04	*P*=0.020	a	1,823	525	$F_{2,17}$=4.110	*P*=0.035	c
N-(methylthio)methylaniline (tentative)	4	3	$F_{2,16}$=1.12	NS		9	5	$F_{2,17}$=1.196	NS	
Benzothiazole	7	7	$F_{2,16}$=0.41	NS		10	13	$F_{2,16}$=0.865	NS	
Methyl (methylthio)methyl disulfide	5	4	$F_{2,13}$=0.44	NS		12	7	$F_{2,16}$=0.469	NS	
Total	10,585	5,862	$F_{2,16}$=5.91	*P*=0.012	a	29,325	20,232	$F_{2,17}$=0.438	NS	

a denotes $P<0.05$ in comparison with B6.6J, b denotes $P<0.05$ in comparison with B6.AK, c denotes $P<0.05$ in comparison with B10.BR, and d denotes $P<0.05$ in comparison with B6.AK and B10.BR. Sharp signs meant that ANOVA was not conducted because of the small number of data points

NS = not significant

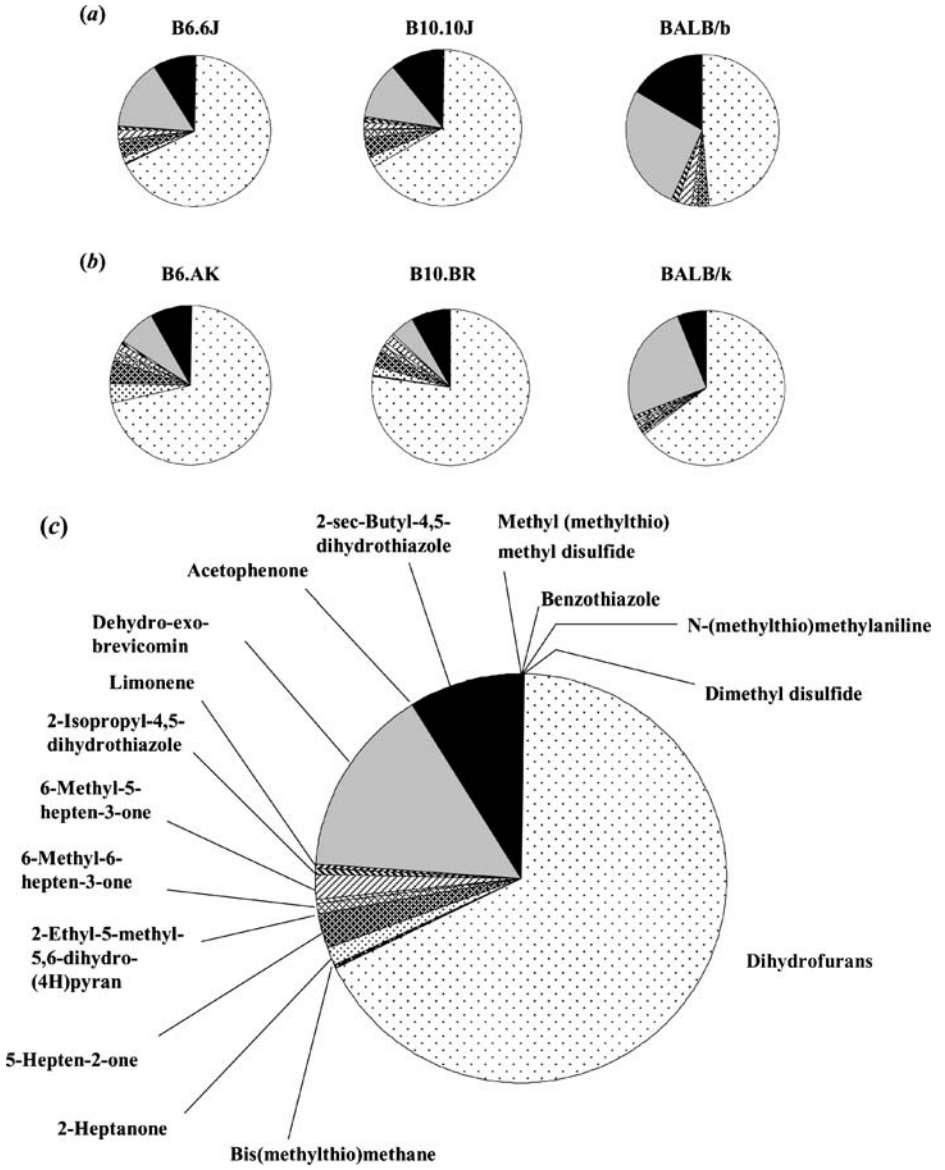


Fig. 5 Comparison of the relative percentages of components in the strains with the same MHC haplotypes H^b **(a)** or H^k **(b)**. The names of the compounds are shown in **(c)**

congenics of the BALB strain. The genetic differences in the region other than the MHC are thus suggested to influence both the amounts and relative percentages of each compound.

Comparison Between B6.6J and Its Mutant Strains In the next part of our experiments, B6.6J strain and its mutants, B6.Tap1 and B6.B2m, were compared. In the mutant B6.B2m, few, if any, MHC class I proteins are expressed on the cell surface (β2 microglobulin "knockouts"), and in the mutant B6.Tap1, the mice lack intracellular transport of peptides to

MHC class I molecules (transporters associated with antigen-processing knockouts). Here, there were significant differences in the amounts of three components: 2-heptanone ($F_{2,15}$= 6.75, P=0.008), 5-hepten-2-one ($F_{2,15}$=9.81, P=0.002), and 3-hepten-2-one ($F_{2,15}$=3.80, P=0.046). The differences in amounts between the host strain and its mutants were especially large in 2-heptanone and 5-hepten-2-one, and Tukey's *post hoc* tests revealed that there were differences between B6.B2m and its host, and between B6.B2m and the other mutant strain (2-heptanone: B6.6J vs. B6.B2m, P=0.04; B6.B2m vs. B6. Tap1, P=0.011; 5-hepten-2-one: B6.6J vs. B6.B2m, P=0.016; B6.B2m vs. B6.Tap1, P=0.002). These differences were due to the smaller amounts in B6.B2m (Fig. 6). This suggests that, even without the stable assembly and intracellular transport of MHC class I molecules, B6.Tap1 may have an odor similar to its host strain B6.6J, whereas B6.B2m mice have less 2-heptanone and 5-hepten-2-one when compared to their host strain because of the lack of MHC class I gene expression. These results, in turn, suggest that the MHC gene complex encoding class I proteins plays an important role in determining the amount of 2-heptanone and 5-hepten-2-one.

Serum Chemical Profiles and Influence of Antibiotic Treatment The serum volatiles' profiles contained some of the same compounds found in the mouse urinary profiles. A dihydrofuran (decomposition product of a pheromone 6-hydroxy-6-methyl-3-heptanone (Novotny et al., 1999b)), DHB, and SBT were observable at low levels in serum (Fig. 7). Also, after the antibiotic treatment, we did not find any significant differences in the profiles of urinary volatile compounds, such as a decrease of components that might be expected as bacterial products.

Discussion

General Considerations Our data support the hypothesis that differences in volatile compound concentrations associated with different individuals or groups can potentially be "quantified" by the vomeronasal (VN) organ or the major olfactory epithelium. Volatile profile constituents recorded in this study from the urinary specimens of adult male mice consisted of several previously identified male pheromones, which are also differentially bound by major urinary proteins (Sharrow et al., 2002), and known to provide quantitative responses of the VN neuronal mechanism for the volatiles that they transport to scent marks. The overall metabolic relationship of these volatiles to MHC genes still remains elusive. Numerous identified compounds can be traced to known metabolic sources, such as the oxidative pathways or production triggered by hormone levels. We found that some of these compounds were detectable in blood serum. However, there was no evidence that their levels in urine were affected by antibiotics, which does not support the microflora hypothesis, without necessarily ruling it out. Within the dozens of urinary volatile constituents, we found numerous compounds influenced by MHC genes, regardless of the host strain. Even with subtle and small differences in genotype (bm mutants), these changes in urinary constituents were easily discernable by our analytical procedures, as well as by the olfactory receptors of mice.

The chemical structures of the volatile urinary constituents identified in our study can be classified into different chemical groups (Fig. 3). However, the metabolic origin of these compounds is mostly unclear. Whereas it is evident from numerous biological experiments that MHC genes influence odor, there is no obvious connection between the direct gene products, class I and class II proteins, and the volatile compounds identified in this work. While the commensal microflora might be a possible source, we found no major changes in

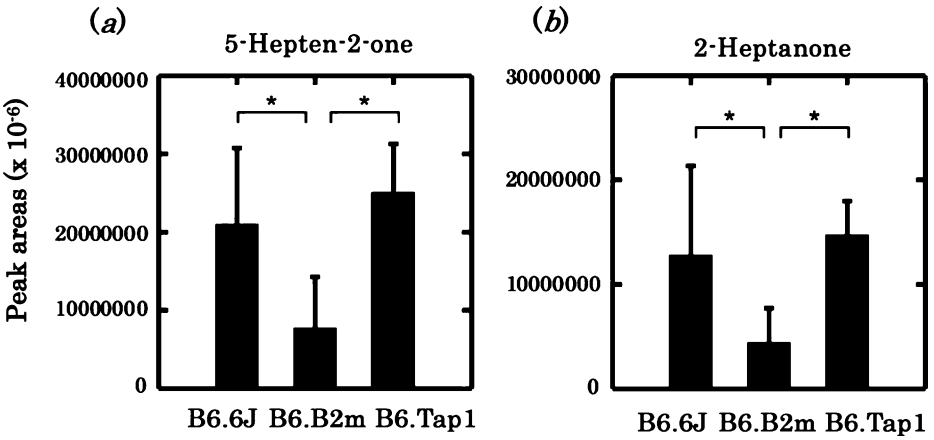


Fig. 6 Comparison of the average total peak areas (±SD) of B6.6J and its knockout mutant strains. **a** 5-hepten-2-one, **b** 2-heptanone. *Asterisks* denote $P<0.05$

the profile of urinary volatiles following treatment with a broad-spectrum antibiotic. Moreover, several major constituents implicated here in the MHC distinction were previously shown to occur in the bladder urine (Novotny et al., 1990), and, as demonstrated here, the same compounds were also detectable in blood serum.

Biochemical Attributes of Detected Volatiles It was previously observed that only urine from anestrous females provided reliable, measurable volatile signals, and the components

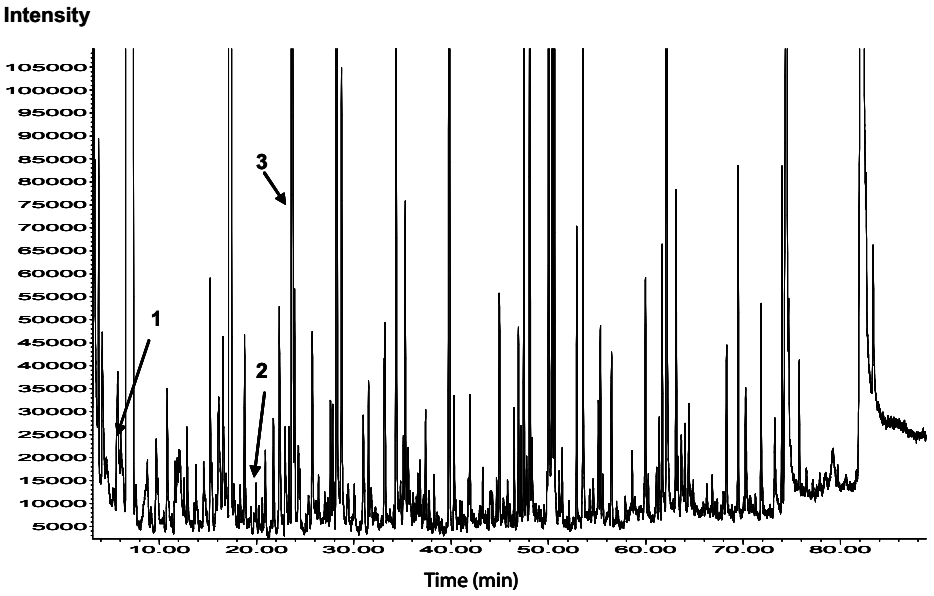


Fig. 7 Volatile compound profile of male mouse serum (B6.6J) determined by GC-MS. Analytical conditions are described in text. *Arrows* indicate the compounds found also in mouse urine: *1*, DHF; *2*, DHB; and *3*, SBT

enhanced during the estrus could "overshadow" the possible changes due to MHC (Schwende et al., 1984). The volatiles enhanced during the estrous cycle (Andreolini et al., 1987) include 2-heptanone, (*E*)-4-hepten-2-one, DHB, and DHF, with the last one implicated as the dehydration artifact (Harvey et al., 1989) of a physiologically active 6-hydroxy-6-methyl-3-heptanone (Novotny, 2003).

The results presented here indicate that several testosterone-dependent urinary volatiles are influenced by MHC, including SBT, DHB, and the above-mentioned DHF. All three major urinary constituents have been implicated as multimodal pheromones (for a review, see Novotny et al., 1999a), with SBT being a uniquely male substance and the other two detectable in large amounts in urine from male mice and only as minor components of the female urine. Most of these substances bind to the major urinary proteins with variable affinities (Sharrow et al., 2002). The metabolic origin of these "mouse-unique" biologically active compounds has not been determined, although it was postulated (Novotny et al., 1995) that the formation of SBT could involve the amino acids L-cysteine and L-isoleucine. It is interesting to note that the MHC appears to influence hormonal pathways (Klein, 1978; Ivanyi, 1978).

The remaining components implicated here as significant in MHC distinction can be divided into two chemically distinct groups: sulfur-containing compounds and carbonyl-containing metabolites. The metabolic origin (and, possibly, hormonal dependence) of IPT is likely similar to that of the male mouse pheromone, SBT, except for a different amino acid origin. Dimethyl disulfide and bis(methylthio)methane, both sulfur-containing molecules, were statistically significant in terms of MHC distinction, whereas methyl (methylthio) methyl disulfide, benzothiazole, and tentatively identified *N*-(methylthio)methylaniline were not, although they might have some other (non-MHC) chemosignaling function.

The biological carbonyl compounds, observed as MHC-correlated in this work, include most prominently 2-heptanone (known to be physiologically active; Novotny et al., 1986; Jemiolo et al., 1989), 6-methyl-5-hepten-3-one, 5-hepten-2-one, and 3-hepten-2-one. Two of these were particularly evident in the distinction between the B6.6J host strain and its two mutant variants. The metabolic origin of these ketones is most likely from oxidation of lipid or fatty acid precursors.

A previous study using a gas sensor array, the so-called "electronic nose" (Montag et al., 2001), was able to distinguish among MHC congenic and class I mutant strains, probably on the basis of very volatile odors due to the analytical procedure used. In their experiment, milliliter volumes of sample headspace (without preconcentration) were used, thus limiting detection to very volatile compounds and possibly major mixture components of the odor. This is consistent with the quantitative differences in the early-eluting components observed in our previous GC studies (Schwende et al., 1986; Novotny et al., 1999b). Moreover, the chemical nature of some of these compounds (oxygenated and sulfur-containing substances) appears favorable for the discriminatory response of gas-sensing surface reactions (Albert et al., 2000). Whereas some of the identified molecules (of both prerenal and postrenal origin) are probably detected by VN neurons (Leinders-Zufall et al., 2000), less volatile and nonvolatile molecules, such as MHC class I peptides (Leinders-Zufall et al., 2004), could also play a role in biological recognition under ecologically different circumstances (direct physical contact with sample). Whether or not there is a connection between MHC-dependent volatile odorants and MHC class I peptides is unknown.

Our study suggests that 2-heptanone and 5-hepten-2-one (products of oxidative pathways) are among the major candidates for chemosignaling that are mediated by the MHC. However, our results also indicate that concentrations of these compounds are not solely determined by MHC because there were differences seen in their amounts even in the

strains with the same MHC, but with differences in other gene regions (different background strains). Thus, it will be necessary to search for candidate genes that might be linked to genes in the MHC region, and that might work together to produce the odor profile characteristic of each individual.

Acknowledgments This work was sponsored partially by the Metabolomics and Cytomics Initiative at Indiana University, Bloomington, IN, and ARO Contract DAAD19-03-1-0215. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the United States Government. We thank Jon N. Laws and Russell Schmidt for valuable advice and help with the antibiotic treatment; K. Musolf for reading a draft; and E. Porche, A. Katzer, and R. Hengsberger for their clerical assistance.

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J Chem Ecol (2007) 33:435–440
DOI 10.1007/s10886-006-9240-7

RAPID COMMUNICATION

Solitary Foraging in the Ancestral South American Ant, *Pogonomyrmex vermiculatus*. Is it Due to Constraints in the Production or Perception of Trail Pheromones?

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Received: 15 March 2006 / Revised: 30 October 2006 / Accepted: 27 November 2006 /
Published online: 23 December 2006
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Abstract Several North American species of *Pogonomyrmex* harvester ants exhibit group foraging, whereas South American species are exclusively solitary foragers. The composition of the secretions of the poison and Dufour glands in the South American species, *Pogonomyrmex vermiculatus*, were analyzed, and the secretions and their components were tested as trail pheromones in laboratory bioassays. The major compounds in the poison gland were the alkylpyrazines, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine. The Dufour gland contained five alkanes, from tridecane to heptadecane, with pentadecane being most abundant. In behavioral bioassays, poison gland extracts and the mixture of pyrazines produced a trail pheromone effect, whereas the Dufour gland extracts and the alkanes had no effect on ant locomotion. We conclude that group foraging in *P. vermiculatus* does not arise from the inability to produce or detect possible pheromones, but rather, from physiological and/or ecological factors.

Keywords Harvester ants · Trail pheromones · Alkylpyrazines · Trimethylpyrazine · Pentadecane · Behavioral bioassays

Introduction

Harvester ants of the genus *Pogonomyrmex* are common in arid and semiarid habitats from southern Canada to the southern extreme of Tierra del Fuego (Taber, 1998). Phylogenetic reconstructions of the genus *Pogonomyrmex* based on morphological, chromosomal, ecological, and behavioral characters show the ancestral nature of the South American species (Taber, 1998). They are exclusively solitary foragers, in contrast to the North American species, some of which exhibit group foraging (a derived character) and generate and use trails for the exploitation of food patches (Johnson, 2001). In this latter group, group

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foraging behavior with recruitment of individuals is mediated by semiochemicals emitted from the poison and Dufour glands (Hölldobler et al., 2001). The major compounds secreted by the poison gland of species from North America are alkylpyrazines (Hölldobler et al., 2001), whereas the Dufour gland contains many types of compounds such as alkanes, alkenes, esters, terpenes, ketones, and aldehydes (Hölldobler et al., 2004). In this work, we tested whether the solitary foraging behavior of an ancestral species is due to its inability to produce or to detect chemical signals involved in group foraging. In the South American species, *Pogonomyrmex vermiculatus*, we identified the chemicals produced by the poison and Dufour glands and evaluated the effect of extracts of both glands and the chemicals contained therein on the locomotion behavior of the ants in laboratory bioassays.

Methods and Materials

Sample Collection Forager workers from five colonies were collected during January 2005 in Las Chinchillas National Reserve (31°30′ S, 71°06′ W). The ants were held in acrylic boxes and transported to the laboratory, where they were maintained according to standard rearing conditions (Hölldobler and Wilson, 1990).

Thirty worker ants of *P. vermiculatus* were killed by freezing (−18°C for 5 min). The poison and Dufour glands were separately dissected from each individual under distilled water by using a stereomicroscope. The individual glands were sealed in soft glass capillaries, which were introduced directly into the injector port of a gas chromatograph by using a solid sample injector as described by Morgan (1990).

GC-MS Analysis of Samples The analyses of compounds contained in the glands were carried out on an HP-5890 gas chromatograph coupled to an HP-5972 mass selective detector with an integrated data system. Ionization by electron impact (70 eV) was carried out at 280°C. The GC was equipped with a SPB-5 capillary column (30 m×0.25 mm ID, Supelco, Deerfield IL, USA). Helium was used as carrier gas at 2 ml/min. The capillaries containing the glands were heated in the injector port to 180°C for 3 min before crushing them in the solid sample injector. The oven temperature was held at 30°C for 2 min, then increased to 250°C at 7°C/min, then held at 250°C for 5 min. The presence or absence of a given compound in the chromatographic profile of each individual gland was determined by comparison of mass spectra with a library database and was confirmed by injection of authentic standards and comparison of Kovats indexes. This procedure was followed for alkanes, 2-tetradecanol, 2,5-dimethylpyrazine (DMP), and 2,3,5-trimethylpyrazine (TMP) (Aldrich Chem. Co.), and for 3-ethyl-2,5-dimethylpyrazine (EDMP) (Carlos Cramer Productos Aromáticos S.A.C.I., see below for details). When standards were not available (heptadecene), tentative identification was based on comparison of the mass spectrum with a library mass spectrum and reported Kovats index values. Identifications were considered positive if the similarity index between experimental and library mass spectra was higher than 90% and if Kovats indexes did not differ by more than 5 U (differences were typically less than 3 U). In the case of ethyldimethylpyrazine, where mass spectra are similar between isomers, an authentic standard that contained 3-ethyl-2,5-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine was analyzed. These two compounds produced well-separated peaks, and the peak with the lower Kovats index, which was identified as 3-ethyl-2,5-dimethylpyrazine (E.D. Morgan, personal communication), coincided with the peak in the

samples analyzed. To quantify the pyrazines and alkanes present in single glands, calibration lines were generated with serial dilutions of authentic standards.

Bioassays with Gland Extracts Poison and Dufour glands were dissected from 40 worker ants. All glands of the same type were deposited in a sample vial containing 1.0 ml hexane, crushed, and filtered to produce extracts. Behavioral tests were performed in a Y-shaped Plexiglass olfactometer with 5×5 cm cross-section, 40-cm long stem, and 15-cm long branches at an angle of 30°. The floor of the olfactometer was lined with filter paper. Chemical trails extending from the base of the stem to the end of the branches were produced with 25 μl of gland extract (one-gland equivalent, treatment trail) and 25 μl of solvent (hexane, control trail). In the stem, chemical trails were drawn along imaginary lines 1.25 cm distant from the walls; in the branches, chemical trails were drawn along the center of the floor. Twenty-five seconds after application of the extracts and solvent, a test ant was liberated at the base of the stem. The first choice of arm was recorded, and the time allocated to this first preference was determined for each test ant. Twenty-four replicates for the poison gland treatment and 26 replicates for the Dufour gland treatment were performed. Additional bioassays were performed with one-gland equivalent of mixtures of the three pyrazine standards (45 ng, 40 replicates) and the five alkane standards (675 ng, 34 replicates), in the same proportions as those found in the glands. The arena was thoroughly cleaned with ethanol between replicates, and the filter paper liner was replaced. Data on the first choice of arm of the Y-olfactometer were analyzed by using a Chi-square test with one degree of freedom and Yates' correction factor; values for residence time in the first arm chosen by the ants were compared with *t*-tests for independent samples (Statistica 6.0 software). For both statistical tests, $\alpha=0.05$.

Results

The poison glands of *P. vermiculatus* contained a mixture of three alkylpyrazines: 2,5-dimethylpyrazine (DMP), 2,3,5-trimethylpyrazine (TMP), and tentatively identified 3-ethyl-2,5-dimethylpyrazine (EDMP). TMP was most abundant (67.7±3.1%, mean ± standard error), followed by DMP (20.1±4.2%), and EDMP (12.2±2.6%). The Dufour glands contained mainly five alkanes (96.9±0.6%: tridecane, tetradecane, pentadecane, hexadecane, and heptadecane), traces of 2-tetradecanol (0.1±0.01%), and a heptadecene (0.1±0.01%). Pentadecane was by far the major component (95.2±0.6%), followed by heptadecane (2.3±0.3%), tetradecane (1.4±0.2%), tridecane (0.9±0.2%), and hexadecane (0.2±0.02%).

In choice bioassays, significantly more worker ants went into the olfactometer arm containing the poison gland extract than the solvent control ($\chi^2=4.26$, $N=15$, $P<0.05$; Fig. 1a), and foragers spent more time in the arm with the poison gland extract than in the arm with the hexane control ($t=2.62$, $df=28$, $P<0.05$; Fig. 1c). This pattern was confirmed in tests that used authentic pyrazine standards, with ants preferring the treatment over the control arm ($\chi^2=4.32$, $N=28$, $P<0.05$; Fig. 1b). Ants also spent more time in the treatment than in the control arm ($t=2.93$, $df=54$, $P<0.05$; Fig. 1d). In contrast, ants showed no preference for the arm with Dufour gland extract (treatment=10, control=5, nonresponders=11; $\chi^2=1.06$, $P=0.30$), and spent equivalent amounts of time in the Dufour gland extract and hexane control arms (252±62 vs. 210±72 sec in the arm containing Dufour gland extract and solvent, respectively; $t=0.44$, $df=28$, $P=0.66$). Experiments with alkane standards confirmed these results. Thus, ants showed no preference for the arm with the alkane mixture vs. solvent

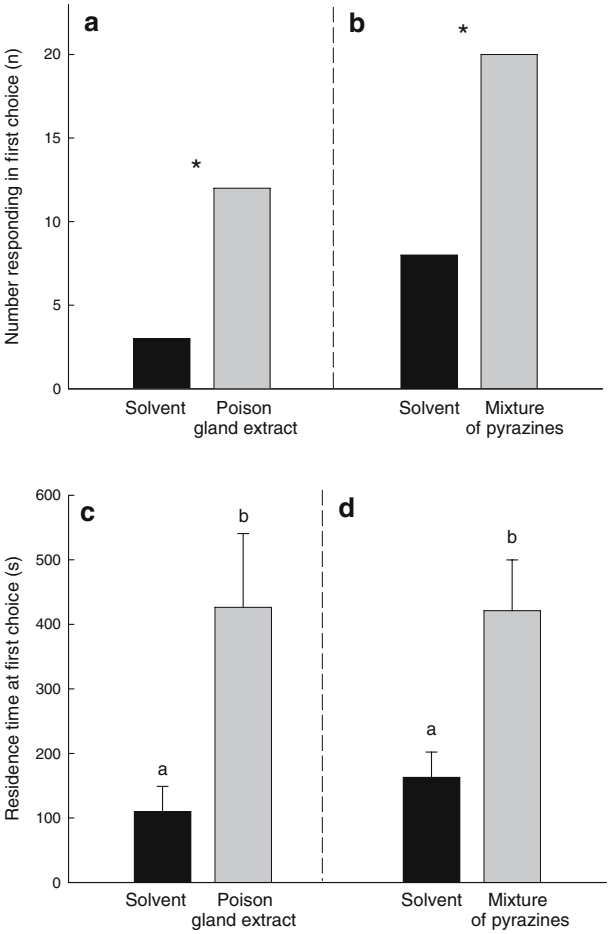


Fig. 1 **a**,**b** First arm choice of *Pogonomyrmex vermiculatus* workers exposed in a Y-olfactometer to different treatments vs. hexane solvent controls (*$P<$ 0.05, Chi-square test). **c**,**d** Time allocated to the arm of the first choice (values are means ± standard error; different letters above bars indicate significant differences in a *t*-test for independent samples)

(treatment=14, control=8, nonresponders=12; $\chi^2=1.14$, $P=0.28$) and spent similar amounts of time in the treatment and control arms (283±38 vs. 290±46 sec in the arm containing the mixture of alkanes and solvent, respectively; $t=0.13$, $df=42$, $P=0.89$).

Discussion

In group-foraging North American species of *Pogonomyrmex*, poison gland secretions constitute short-lived signals (20 to 30 min) allowing ants to recruit nestmates and move long distances towards food patches along trunk trails (see references in Hölldobler et al., 2001). These secretions contain pyrazines, which have been identified as trail pheromone components in several genera of the subfamily Myrmicinae (*Aphaenogaster*, *Atta*, *Eutetramorium*, *Manica*, *Messor*, *Myrmica*, *Pheidole*, *Pogonomyrmex*, and *Tetramorium*) (Hölldobler et al., 2001, and references therein). The poison gland of *P. vermiculatus* contained ca 45 ng pyrazines per gland, which is within the range reported for other

Pogonomyrmex species (30–200 ng per gland) (Hölldobler et al., 2001). Furthermore, our bioassays (Fig. 1 a–d) suggest that compounds present in the poison gland of *P. vermiculatus* can function as trail pheromones. Finally, the preference of treatment (poison gland extract or mixture of pyrazines) over control was ca 3:1 in the first choice of arm and also in the residence time. These proportions are comparable with the strength of recruitment behaviors elicited by pyrazines in group foraging *Pogonomyrmex barbatus* (ratio ca 4:1 for treatment vs. control; Hölldobler et al., 2001).

The secretions from the Dufour gland of group-foraging North American *Pogonomyrmex* serve as long-lasting orientation signals (on the order of hours) and allow continuous reinforcement of the trail leading to the foraging site with chemical signals specific for each colony (Hölldobler et al., 2004, and references therein). Hydrocarbons have been reported as the principal compounds secreted by the Dufour gland in the genus *Pogonomyrmex* (Hölldobler et al., 2004). Pentadecane, the major compound present in *P. vermiculatus*, has also been reported as the major compound in the Dufour glands of *Pogonomyrmex occidentalis* and *Pogonomyrmex salinus* (Billen et al., 1987, do Nascimento et al., 1993), two group-foraging species. However, neither the Dufour gland extract nor the reconstructed hydrocarbon mixture contained therein elicited changes in the locomotion of *P. vermiculatus* in relation to controls. This lack of effect may be due to the fact that *P. vermiculatus* only uses food patches situated near the nest (at distances <5 m, H. Torres-Contreras, personal observation), and hence, the reinforcement of trails with chemical signals would be superfluous. However, olfactometry tests do not allow us to exclude the possibility that in the field, these compounds may function as homing signals (see references in Hölldobler et al., 2004).

In this study, we demonstrated that *P. vermiculatus*, a solitary foraging harvester ant, produces and perceives compounds that function as trail pheromones in congeneric species. Given the ancestral nature of *P. vermiculatus*, this suggests that the character "production of semiochemicals" was fixed early in the evolutionary history of the group. If *P. vermiculatus* is able to perceive chemicals that in other *Pogonomyrmex* species are involved in group foraging behavior, then why does not this species (or, in general, the species of South America) exhibit group foraging? Several explanations are possible. First, it is probably not a question of the amount of compounds deposited by foragers because the total content in the glands of *P. vermiculatus* was similar to that of group foraging *Pogonomyrmex* species, and the content of one poison gland was shown to affect the locomotion patterns of test ants. Second, the absence of group foraging may be related to the number of foraging individuals. If the number of foragers is low, then, their efficiency in distributing the trail pheromones in the substratum would also be low, thus, constituting a limitation to the amplification of the signal (e.g., Beekman et al., 2001). In support of this proposal, the nests of the South American *Pogonomyrmex* species have fewer workers than the nests of North American species (Taber, 1998), and therefore, fewer individuals are assigned to foraging tasks (H. Torres-Contreras, personal observation). Third, because the number of ant species that forage and compete for seeds in arid habitats of Northern Chile is low (Medel, 1995) in comparison with the number of species present in the deserts of North America (Johnson, 2001, and references therein), group foraging may not be necessary in the South American species. Fourth, the proportion at which the three pyrazines were found in *P. vermiculatus* was similar to that found in *Pogonomyrmex maricopa*, a species documented as a solitary forager but which when confronted with a high abundance of resources exhibits recruitment of individuals and group foraging (Johnson, 2001). Thus, the pyrazines present in the poison gland of *P. vermiculatus* could play a role as a trail

pheromone and allow nestmate recruitment when there is a high availability and a patchy distribution of seeds in the habitat. The testing of these hypotheses is currently underway.

Acknowledgments We thank E. D. Morgan for advice during the implementation of the solid sample injection technique and for sharing unpublished results on the analysis of pyrazines and J. G. Millar for suggestions during the editing of the original manuscript. We also thank Claudia Cabrillana for assistance in the laboratory. Financial support by FONDECYT (grant 3060064 to H.T.-C.) and International Program in the Chemical Sciences at Uppsala University (H.M.N.) is gratefully acknowledged.

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J Chem Ecol (2007) 33:441–445
DOI 10.1007/s10886-006-9237-2

RAPID COMMUNICATION

Flower Scent of Floral Oil-Producing *Lysimachia punctata* as Attractant for the Oil-Bee *Macropis fulvipes*

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Received: 20 October 2006 / Revised: 7 November 2006 / Accepted: 27 November 2006 /
Published online: 7 December 2006
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Abstract Most flowers offer nectar and/or pollen as a reward for pollinators. However, some plants are known to produce mostly fatty oil in the flowers, instead of nectar. This oil is exclusively collected by specialized oil-bees, the pollinators of the oil-plants. Little is known about chemical communication in this pollination system, especially how the bees find their hosts. We collected the floral and vegetative scent emitted by oil-producing *Lysimachia punctata* by dynamic headspace, and identified the compounds by gas chromatography coupled to mass spectrometry. Thirty-six compounds were detected in the scent samples, several of which were flower-specific. Pentane extracts of flowers and floral oil were tested on *Macropis fulvipes* in a biotest. Flower and oil extracts attracted the bees, and some of the compounds identified are seldom found in the floral scent of other plants; these may have been responsible for the attraction of the bees.

Keywords Floral and vegetative scent · Dynamic headspace · GC-MS · Oil-flower oil-bee pollination system · *Lysimachia punctata* · *Macropis fulvipes* · Flight cage · Biotest

Introduction

The interaction between flower-oil-producing plants and flower-oil-collecting bees is an interesting pollination system, and was first described by Vogel (1971). About 2,400 plant species in 10 different families produce flower oil by specific organs (elaiophores), mostly instead of nectar. The oil is the main floral reward, and is collected by ca. 320 species of specialized bees that pollinate the flowers (Neff and Simpson, 2005). The collected oil is used together with pollen as provision for the larvae, and some species use it also as a brood-cell lining (Cane et al., 1983). Oil-producing flowers and flower-oil-collecting bees are mostly neotropical. In Europe, only some species of *Lysimachia* (Myrsinaceae) produce floral oil, which is collected only by *Macropis* bees (Mellitinae) (Vogel, 1986). *Macropis* collects oil and pollen only from *Lysimachia* spp. as food for their larvae. Little is known

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about how the bees find their host plants, but floral scents are assumed to be responsible for the attraction of *Macropis* to *Lysimachia* (Vogel, 1986). From other pollination systems, it is known that floral scent is important in attracting the pollinators (e.g., Dötterl et al., 2005, 2006).

The purpose of this study was to collect and identify the scent emitted by the green parts, flowers, and pure oil of *Lysimachia punctata* L. by dynamic headspace and gas chromatography coupled to mass spectrometry (GC-MS) methods. Further, solvent extracts of flowers were tested for their attractivity to *Macropis fulvipes* (Fab.) in a biotest conducted in a flight cage.

Methods and Materials

Plant Material and Volatile Collection Floral scent was collected from plants growing in the Ecological–Botanical Garden of the University of Bayreuth. Scent was collected from flowering inflorescences (3 samples, 1–3 stems, 15–35 flowers), from pure oil (3 samples from 50 flowers each), and from nonflowering stems (3 samples, 3–5 stems). Oil for scent analyses was collected on filter paper. To trap scent, the oil-containing filter paper and the flowering, and nonflowering stems were enclosed for 10 min (flowering stems) or 60 min (nonflowering stems and oil) within polyester oven bags (31×20 cm for flowering and nonflowering stems; 10×10 cm for pure oil, Toppits®, Germany), and the emitted volatiles were trapped for 2–4 min in an adsorbent tube by using a membrane pump (G12/01 EB ASF Rietschle-Thomas Inc., Puchheim, Germany), adjusted to a flow rate of 200 ml/min. ChromatoProbe quartz microvials from Varian Inc. (length 15 mm, inner diam. 2 mm) were used as adsorbent tubes after cutting the closed end, and filling them with a mixture (1:1) of 3 mg Tenax-TA (mesh 60–80, Supelco) and Carbotrap (mesh 20–40, Supelco). The adsorbents were held in the tubes with glass wool.

To obtain samples for biotests, 50 flowers (two samples, each flower was extracted for 1 min) were extracted in pentane p.a. (Merck KgaA, Germany). Furthermore, floral oil was collected with filter paper from 50 flowers and dissolved in pentane p.a. (one sample).

Chemical Analysis The dynamic headspace samples were analyzed on a Varian Saturn 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector fitted with the ChromatoProbe kit, using the same method as described by Dötterl and Jürgens (2005). The GC-MS data were processed by using the Saturn software package 5.2.1. Component identification was carried out with the NIST 02 mass spectral database, or MassFinder 3, and confirmed by comparison of retention times with published data (Adams, 1995). Identification of individual components was confirmed by comparison of both mass spectrum and GC retention data with those of authentic standards. For quantification, known amounts of different terpenoids, fatty acid derivatives, and benzenoids were injected, and the mean response of these compounds was used.

Biotests Pentane extracts of flowers and pure oil were used in biotests conducted in a flight cage (7.2×3.6×2.2 m) in which ca. 20 female bees built nests and collected oil and pollen as food for their larvae. All bees in the flight cage were individually marked, and before the tests, all *Lysimachia* plants were removed. Two setups were used for the tests. First, a dual choice test in which a pentane extract of 50 flowers of *L. punctata* (corresponding to flowers of 3–4 flowering stems) and pure pentane (control) were offered to the bees on yellow paper flowers. Second, a three-choice test in which a pentane extract of 50 flowers,

a pentane extract of oil collected from 50 flowers, and pure pentane were offered to the bees on a horizontal glass surface (diam. 2.5 cm). The responses of bees were observed for 1 hr, and bees landing on the artificial "flowers" were recorded. The responses of bees were classified into two categories: (1) landing, when the bees landed on the artificial flowers; and (2) collection of oil, if the bees collected oil for at least 2 sec from the artificial "flowers." The oil collection behavior of *Macropis* was previously described in detail by Vogel (1986).

Results and Discussion

The chemical composition of scent emitted by flowering and nonflowering stems and from pure floral oil of *L. punctata* is summarized in Table 1. In total, 36 compounds were found—among them fatty acid derivatives, aromatics, mono- and sesquiterpenoids. The total amount and the number of compounds varied greatly between flowering and nonflowering stems. The vegetative parts of the plants emitted 14 compounds, especially several fatty acid derivatives and terpenoids—among them (*E,E*)-α-farnesene, (*Z*)-3-hexenyl acetate, D-limonene, and the benzenoid benzaldehyde. Most compounds were emitted by the flowering stems (32). Twenty-two of these compounds were not found in the samples collected from the nonflowering stems, and these were emitted only by the flowers. Most of the flower-specific compounds were found only in small percentages, and only four compounds occurred with amounts of at least 5%, i.e., 1-phenyl-1,2-propanedione, linalool, (*E*)-citral, and (*Z*)-β-ocimene. Some compounds, which were found in nonflowering and flowering stems, were emitted in small amounts from vegetative parts, but with high total and percentage amounts from flowering stems [e.g., (*E*)-β-ocimene (*Z*)-3-hexenyl acetate]. The oil samples emitted nine compounds (four of them were not emitted from nonflowering stems) with high percentage amounts of aromatics 1-phenyl-1,2-propanedione and 1-hydroxy-1-phenyl-2-propanone. These aromatic compounds are uncommon floral scent compounds, and are described in the floral scent of only few species (see Knudsen et al., 2006).

The biotests revealed that the compounds emitted by the flower and oil extracts were responsible for attraction of *M. fulvipes* (Table 2). In the two-choice test, 12 of the ca. 20 nest-provisioning females were attracted to the paper flower impregnated with a pentane extract of *L. punctata* flowers from a distance of several meters. No bees were attracted to the control flower. In the three-choice test, 11 of the ca. 20 nest-provisioning individuals landed on the glass surfaces containing either the pentane extract of whole flowers or the pentane extract of pure oil. None was attracted to the control glass surface. Eight of the 11 attracted individuals landed on the glass surface containing the whole flower extract, and 2 of them collected oil (1 of these specimens for several minutes). Three individuals were attracted to the "oil flower," and one of these collected oil from the surface (for several minutes). In this three-choice test, oil bees were attracted by the flower (floral oil) scent without any visual cues (flower shape or color). Dynamic headspace analyses from the artificial flowers revealed that they released nearly the same spectrum of compounds emitted from flowers and pure oil of *L. punctata* (Schäffler and Dötterl, unpublished data).

The results reveal that vegetative parts and flowers of *L. punctata* emit several scent compounds, and that some of them are also emitted from pure oil. Because these oil compounds were emitted in much higher total amounts from whole flowers compared to pure oil (Table 1), it is suggested that the scents that are released by floral tissues are taken up by the oil (because they are soluble in it) and are revaporized (compare with Raguso,

Table 1 Mean percentage (%±SE) and total amount of scent compounds emitted by nonflowering and flowering stems, and by pure oil of *L. punctata*

	Nonflowering Stems	Flowering Stems	Floral-Oil
Total Amount of Scent (Mean±SE) and (Flowering) Stem, ng/hr	2.0±0.7	516.9±242.6 (67.1±17.9)[a]	6.5±2.0 (5.7±2.0)
Fatty acid derivatives			
(*Z*)-3-Hexen-1-ol[b]	1.1±0.5	3.6±1.5	0.6±0.5
(*Z*)-3-Hexenyl acetate[b]	18.3±2.4	29.9±11.5	1.5±1.3
(*Z*)-3-Hexenyl butyrate[b]	–	0.1±0.03 (0.4±0.04)	–
(*Z*)-3-Hexenyl isovalerate	1.9±0.2	0.3±0.1	–
(*Z*)-Jasmone	1.9±0.6	–	–
(*E*)-Geranylacetone[b]	4.2±0.5	0.4±0.1	3.5±1.7
(*E*)-2-Dodecenal[b]	–	0.1±0.04 (0.4±0.1)	0.2±0.1 (0.2±0.2)
2-Tridecanone[b]	–	0.3±0.1 (1.3±0.2)	1.2±0.4 (1.4±0.6)
Aromatics			
Benzaldehyde[b]	12.1±2.5	4.5±1.8	8.5±3.2
1,4-Dimethoxybenzene[b]	–	0.9±0.2 (3.9±0.5)	–
1-Phenyl-1,2-propanedione[b]	–	14.9±4.3 (54.8±4)	76.9±4.4 (91.7±1.7)
Unknown *m/z* 79,108,80,77,43	–	0.2±0.1 (0.8±0.3)	–
1-Hydroxy-1-phenyl-2-propanone[b]	–	1.0±0.4 (3.5±0.8)	5.5±1.9 (6.7±2.3)
Unknown *m/z* 108,79,43,107,77	–	0.1±0.04 (0.5±0.1)	–
Monoterpenoids			
β-Pinene[b]	–	0.2±0.05 (1.04±0.3)	–
D-Limonene[b]	9.0±1.3	0.3±0.1	–
(*Z*)-β-Ocimene[b]	–	1.5±0.2 (6.4±0.9)	–
(*E*)-β-Ocimene[b]	3.3±0.7	34.0±7.6	–
(*Z*)-Linalool oxide (furanoid)[b]	–	0.1±0.05 (0.8±0.5)	–
Unknown *m/z* 91,107,79,119,135	–	0.4±0.2 (1.6±0.6)	–
Terpinolene[b]	2.5±0.3	0.1±0.03	–
Linalool[b]	–	2.2±0.5 (8.6±0.6)	–
Unknown *m/z* 91,93,77,79,121	–	0.2±0.1 (0.8±0.1)	–
Citronellal[b]	–	0.4±0.1 (1.3±0.2)	–
α-Terpineol	–	0.2±0.03 (0.7±0.1)	–
Verbenone[b]	–	0.5±0.3 (1.8±0.7)	–
(*Z*)-Citral[b]	–	0.4±0.1 (1.4±0.2)	–
(*Z*)-Geraniol[b]	–	0.7±0.3 (2.4±0.4)	–
(*E*)-Citral[b]	–	1.7±0.9 (6.7±2.7)	–
(*E*)-Geranic acid[b]	–	0.3±0.1 (0.9±0.2)	–
Unknown *m/z* 91,39,107,79,77	–	0.03±0.02 (0.1±0.05)	–
Sesquiterpenoids			
Unknown *m/z* 91,79,119,77,93	3.2±1.1	–	–
β-Caryophyllene[b]	0.6±0.3	0.7±0.1	2.0±1.1
(*E*)-α-Bergamotene	0.9±0.2	–	–
Germacrene D[b]	0.4±0.3	–	–
(*E,E*)-α-Farnesene[b]	40.6±6.4	0.2±0.1	–

[a] Values in parentheses indicate the mean percentage (±SE) of compounds emitted from flowering stems and floral-oil, excluding compounds also emitted from nonflowering stems

[b] Identification based on authentic standards

Table 2 Number of females of *M. fulvipes* landing in a dual choice test on paper flowers impregnated with pentane (control) or with a pentane extract of flowers of *L. punctata*, and in a three-choice test on glass surfaces containing a pentane extract of flowers/pure oil of *L. punctata* or pentane only

	Control	Flower Extract	Oil Extract
Dual choice test (paper flowers)	0	12 (0)[a]	nt[b]
Three-choice test (glass surfaces)	0	8 (2)	3 (1)

[a] Values in parentheses are the number of bees collecting oil.

[b] nt = not tested

2004). Furthermore, female bees were attracted to pentane extracts of whole flowers and pure floral oil, and the uncommon floral scent compounds characteristic for *L. punctata* were possibly responsible for this attraction. It is interesting to note that bees only collected oil from the glass surface, and oil-collection behavior was not elicited at the paper flowers. Therefore, it seems that bees have chemoreceptors on their legs for detection of oil, and that oil-collection behavior is triggered when oil, available on a surface, comes in contact with the receptors. Free-standing oil was not available on the paper flowers because it was taken up by the paper.

Acknowledgements We are grateful to Gregor Aas for permission to build the flight cage in a green house at the Ecological–Botanical Garden (EBG), and to use *L. punctata* plants growing in the EBG for this study. Peter Hartmann helped collect bees for the flight cage. Karlheinz Seifert and Thomas Gedig provided a standard 1-hydroxy-1-phenyl-2-propanone. Joseph Woodring, Gregor Aas, and two anonymous reviewers improved earlier versions of this manuscript.

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J Chem Ecol (2007) 33:447–448
DOI 10.1007/s10886-006-9241-6

ERRATUM

(2*s*,12*z*)-2-Acetoxy-12-heptadecene: Major Sex Pheromone Component of Pistachio Twig Borer, *Kermania pistaciella*

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Published online: 9 January 2007
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The following error was published in Table 1 of this article.

An erroneous comma appears in all numbers in the first 10 rows below the column headings DB-23, DB-5, and DB-210. The comma greatly distorts the meaning. The correct Table 1 appears below.

The online version of the original article can be found at http://dx.doi.org/10.1007/10.1007/s10886-006-9190-0.

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Table 1 Retention Indices (RI) of Synthetic Standards, and of Components **A** and **B** in Pheromone Gland Extracts of Female *Kermania pistaciella*

Compounds	RI on GC column		
	DB-23	DB-5	DB-210
A in Fig. 2	2376	1890	2093
B in Fig. 2	2355	2011	2290
B1 (= hydrogenated **B**)	2309	2021	2286
(*Z*)-12-Heptadecen-2-ol	2376	1890	2093
(*Z*)-2-Acetoxy-12-heptadecene	2355	2011	2290
(*Z*)-2-Acetoxy-10-heptadecene	2342	2002	2281
(*Z*)-2-Acetoxy-11-heptadecene	2348	2007	2284
(*E*)-2-Acetoxy-13-heptadecene	2342	2014	2276
(*Z*)-2-Acetoxy-13-heptadecene	2363	2019	2297
Heptadec-1-yl acetate	2450	2105	2386
2-Acetoxyheptadecane (= **B1**)	2309	2021	2286
3-Acetoxyheptadecane[1]	2284	2001	2262
4-Acetoxyheptadecane[1]	2254	1981	2237
5-Acetoxyheptadecane[1]	2240	1970	2226
6-Acetoxyheptadecane[1]	2231	1965	2217
7-Acetoxyheptadecane[1]	2227	1962	2215

[1] Mass spectra of 2-, 3-, 4-, 5-, 6- and 7-acetoxyheptadecane did not have fragment ions diagnostic of the acetoxy position.

J Chem Ecol (2007) 33:449–461
DOI 10.1007/s10886-006-9238-1

Allelochemical Induction of Cytochrome P450 Monooxygenases and Amelioration of Xenobiotic Toxicity in *Helicoverpa zea*

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Received: 14 July 2006 / Accepted: 4 December 2006 /
Published online: 10 January 2007
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Abstract Polyphagous herbivores encounter allelochemicals as complex mixtures in their host plants, and the toxicity of an individual compound may be influenced by the chemical matrix in which it is encountered. Certain plant constituents may reduce toxicity of cooccurring compounds by inducing detoxification systems, including cytochrome P450s, which can metabolize a broad range of substances. The polyphagous corn earworm *Helicoverpa zea* encounters a diversity of plant allelochemicals in its many host plants and, as well, can encounter aflatoxins, mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* that infect damaged grains. Dietary supplementation of each of three plant allelochemicals that are frequently (coumarin, COU), occasionally (indole-3-carbinol, I3C), or rarely (xanthotoxin, XAN) encountered by *H. zea* larvae substantially reduced the toxicity of aflatoxin B1 (AFB1) to *H. zea*. Compared to fourth instars on diets containing 1 μg/g AFB1 that failed to develop and pupate, fourth instars on diets containing I3C and XAN increased in mass by 216.1 and 700% after 6 days, and pupated at rates of 40 and 88%, respectively. Diets containing COU or XAN also significantly reduced the mortality rates of caterpillars exposed to the insecticides, diazinon and carbaryl. Diets containing COU and XAN increased *CYP6B8* transcripts 2.6-fold; *CYP321A1* transcripts increased 20.7, 8.3, and 10.6-fold in response to COU, I3C, and XAN, respectively. These results indicate that consumption of plant allelochemicals can ameliorate toxicity of natural and synthetic toxins encountered by insects, and they suggest that P450s induced by these allelochemicals contribute to detoxification of these chemicals in *H. zea*.

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Keywords *Helicoverpa zea* · Allelochemicals · Aflatoxin B1 · Insecticides · Cytochrome P450 monooxygenase · Indole-3-carbinol · Coumarin · Xanthotoxin

Abbreviations

AFB1	Aflatoxin B1
P450	Cytochrome P450 monooxygenase
DMSO	Dimethyl sulfoxide
I3C	Indole-3-carbinol
XAN	Xanthotoxin
COU	Coumarin
SDS	Sodium dodecyl sulfate

Introduction

Polyphagous herbivores encounter allelochemicals as complex mixtures in their host plants, and the toxicity of an individual compound may be influenced by the chemical matrix in which it is encountered (Berenbaum, 1985). In some synergistic interactions, cooccurring plant constituents interfere with or disable insect detoxification systems to enhance toxicity. In the polyphagous corn earworm *Helicoverpa zea*, for example, the toxicity of the phototoxic plant furanocoumarin xanthotoxin is enhanced in the presence of myristicin, a phenylpropene essential oil component that inhibits the cytochrome P450 monooxygenases that detoxify furanocoumarins in this species (Berenbaum and Neal, 1987). Whereas certain allelochemical interactions are beneficial to the plant in enhancing its defense against herbivores, other interactions among allelochemicals may be beneficial to the herbivore. In some cases, cooccurring chemicals reduce the toxic consequences of allelochemical ingestion in that some herbivores selectively sequester host plant carotenoids and xanthophylls, which reduce oxidative stress produced by ingestion of cooccurring plant phototoxins (Aucoin et al., 1990; Fields et al., 1990; Carroll et al., 1997). Other interactions that benefit the herbivore result from activation or potentiation of detoxification systems that include P450s. A tremendous diversity of plant allelochemicals, including furanocoumarins, coumarin, phenylpropenes, alkaloids, triterpenes, tetraterpenes, flavonoids, and cardenolides are capable of inducing P450 activities in herbivorous insects (Yu, 1982, 1983, 1987; Cohen et al., 1989; Capua et al., 1991; Snyder et al., 1993; Hung et al., 1995; Li et al., 2002a), but the toxicological benefits of such induction have been documented only rarely. In some cases, these benefits are mediated by the plant signaling substances, jasmonic acid and salicylic acid, which activate biosynthetic defense responses in plants damaged by herbivores or pathogens. Ingestion of jasmonic acid or salicylic acid leads to the up-regulation of CYP6B8 and other detoxification enzymes in *H. zea* (Li et al., 2002b). Induction of these broadly substrate-specific enzymes in response to plant signals allows this insect to gear up its capacity to detoxify allelochemicals in advance of their biosynthesis by the plant (Li et al., 2002b).

Because of the broad substrate-specificity of at least some P450s in the polyphagous *H. zea*, the potential exists for interactions between plant allelochemicals and other cooccurring toxins. Whether these interactions benefit the herbivore depends on the substrate specificities and catalytic activities of the induced P450s. Because herbivorous insects are exposed to a variety of toxic xenobiotics other than host plant constituents, Cleveland et al. (2003) have suggested that the ability of insect herbivores to detoxify plant allelochemicals may equip them for resistance against toxins produced by pathogens that infect their host plants. For *H. zea*, one such toxin is aflatoxin B1, a metabolite produced by

Aspergillus flavus and *Aspergillus parasiticus*, two closely related fungal species that can infect multiple host plants of *H. zea* (Diener et al., 1987; Wilson and Payne, 1994), particularly during infestation by this insect (Dowd, 1998). Aflatoxins are dihydrofurocyclopentenones or dihydrofurolactones that owe their toxicity to their ability to form irreversible adducts to nucleic acids with the concomitant inhibition of DNA replication and DNA-dependent transcription (Murray et al., 1982). Unlike most P450-mediated detoxification reactions in which toxins are inactivated, P450-mediated reactions are capable of transforming aflatoxin B1 (AFB1) to more toxic metabolites, including AFB1-8,9-epoxide, by the epoxidation of the terminal furan ring (Murray et al., 1982). Such bioactivation of AFB1 is mediated by CYP6A2 in the Hikone-R strain of *Drosophila melanogaster* (Saner et al., 1996) and several P450s in humans. In *H. zea*, the toxicity of AFB1 is ameliorated by piperonyl butoxide inhibition of P450s (Zeng et al., 2006) but the identity of the P450(s) responsible for this bioactivation has not yet been defined.

In addition to mycotoxins, herbivorous insects can encounter insecticides in combination with allelochemicals. Whether insecticides are detoxified or bioactivated by insect P450s depends both on the insecticide structure and the specific P450 isoforms present. Whereas pyrethroids are detoxified by CYP6D1 in the house fly *Musca domestica* (Scott, 1999; Scott and Wen, 2001) and by CYP6B8 (Li et al., 2004) and CYP321A1 in *H. zea* (Sasabe et al., 2004), bioactivation of the organophosphorothioate pesticide diazinon is mediated by CYP6A1 in the house fly (Sabourault et al., 2001). In some species, the ability to metabolize plant allelochemicals cross-protects them against synthetic insecticides (Kennedy, 1984; Li et al., 2000). The major mechanism for this cross-resistance is the ability of some P450s to detoxify both plant allelochemicals and synthetic insecticides (Li et al., 2004; Sasabe et al., 2004). Some of these P450s are differentially induced by a wide range of host plant allelochemicals, including those that are nontoxic themselves (Prapaipong et al., 1994; Hung et al., 1995; Li et al., 2002a, 2004; Sasabe et al., 2004), thereby influencing the ability of insects to detoxify or bioactivate compounds ingested in their diet.

The broad diet of *H. zea* leads to exposure to a wide range of allelochemicals, which are processed in part by P450s with broad substrate ranges (Li et al., 2004). At the same time, the susceptibility of several of its host plants to *Aspergillus* infection makes this species likely to encounter mycotoxins, and its economic status virtually assures exposure to multiple insecticides throughout its range. Thus, *H. zea* is an ideal subject for investigating the toxicological significance of allelochemical induction of P450s in the presence of exogenous toxins. Specifically, we examined the toxicity of two insecticides, diazinon and carbaryl, and one mycotoxin, AFB1, in the presence of three plant allelochemicals known to induce P450s that are encountered with varying frequency by this species: coumarin (COU), which is frequently encountered, indole-3-carbinol (I3C), which is occasionally encountered, and xanthotoxin (XAN), which is rarely encountered throughout the host plant range of *H. zea* larvae. We also determined the effects of these plant allelochemicals on transcription of the *H. zeaCYP6B8*, *CYP6B9*, *CYP6B27*, and *CYP321A1* genes encoding P450s known to detoxify allelochemicals and insecticides.

Methods and Materials

Chemicals and Enzymes

Aflatoxin B1, indole-3-carbinol, coumarin, and xanthotoxin (8-methoxypsoralen; 99.6% purity) were obtained from Sigma (St. Louis, MO, USA). Analytical grade dimethyl

sulfoxide (DMSO) was purchased from Fisher Scientific. RQ1 DNase, RNasin and AMV reverse transcriptase were from Promega (Madison, WI, USA), Taq polymerase and deoxynucleotide triphosphates (dNTPs) were from Invitrogen (Carlsbad, CA, USA) and the TRIzol reagent was from Gibco-BRL (Cleveland, OH, USA). Diazinon and carbaryl were from Chem Service (West Chester, PA, USA).

Insects

An insecticide-susceptible laboratory strain of *H. zea*, provided by B. Ria Barrido of Abbott Laboratories (Chicago, IL, USA) was used for all experiments. *H. zea* larvae were individually reared on a semisynthetic control diet containing wheat germ (Waldbauer et al., 1984) and maintained in an insectary kept at 23–26°C with a photoperiod of 16-hr light to 8-hr dark. For each experiment, caterpillars that had molted within 12 hr were selected for all treatments to insure homogeneity in developmental age.

AFB1 Bioassays

Stock solutions of AFB1 were prepared in DMSO at a concentration of 1 μg/μl. Caterpillars within 12 hr of molting to either fourth or fifth instar, depending on experiment, were assigned to four different treatments: (1) control diet containing 200 μl DMSO per 100 g; (2) AFB1 diet containing 100 μl of AFB1 stock solution in DMSO and 100 μl DMSO per 100 g diet (i.e., 1 μg AFB1 per gram diet); (3) allelochemical diets containing 100 mg indole-3-carbinol, 100 mg coumarin or 50 mg xanthotoxin dissolved in 100 μl DMSO and additional 100 μl DMSO per 100 g diet (corresponding to 1 mg COU or I3C per gram diet or 0.5 mg XAN per gram diet); (4) allelochemical + AFB1 diets, containing 100 mg indole-3-carbinol or coumarin, 50 mg xanthotoxin (fifth instars) or 25 mg xanthotoxin (fourth instars) dissolved in 100 μl DMSO and 100 μl AFB1 stock solution in DMSO per 100 g diet (corresponding to the combination of 1 μg AFB1 and 1 mg COU or I3C or 0.25–0.5 mg XAN per g diet).

Caterpillars feeding on these different diets were individually reared in 30-ml plastic cups in an insectary at 23–26°C with a photoperiod of 16-hr light to 8-hr dark. Weight gain was measured after 3 d in experiments with fifth instars and 6 d in experiments with fourth instars. Larval developmental stage and mortality were monitored daily until pupation, at which point pupal weights were measured within 48 hr of the final larval molt. Each set of bioassays was replicated three times, and 20 larvae were tested in each bioassay.

Insecticide Bioassays

Diazinon was used in our insecticide bioassays because this insecticide is bioactivated by P450s in some species (Feyereisen, 1999). Fifth instars were reared in an insectary at 23–26°C with a photoperiod of 16-hr light to 8-hr dark for 48 hr on diets containing either COU at 1 mg/g diet, I3C at 1 mg/g diet or XAN at 0.5 mg/g diet, after which time 3 μl of 100 μg/μl diazinon in acetone (300 μg total) and 3 μl of 33.3 μg/μl carbaryl in acetone (100 μg total) were topically applied to each caterpillar. These insecticides were not delivered in the diet because their fast-acting neurotoxic effects would likely interfere with allelochemical ingestion. Control bioassays were conducted with topical applications of 3 μl of acetone. Treated insects were kept in an incubator at 28°C with a photoperiod of 16-hr light to 8-hr dark, and mortality was assessed 5 hr after insecticide application. Insects were considered dead if they were on their backs and unable to right themselves when

disturbed. Bioassays were replicated at least three times and 20 larvae were tested in each bioassay.

RNA Isolations

Fifth instar *H. zea* within 12 hr of molting and within a weight range of 170–270 mg were randomly assigned to treatment groups. Twenty larvae in each treatment group were placed on diets containing COU at 1 mg/g diet, I3C at 1 mg/g diet or XAN at 0.5 mg/g diet. There were two control treatments, one with unamended diet and another with artificial diet containing 0.2% DMSO to control for solvent effects. After 48 hr of feeding, larvae were sacrificed and midguts excised and cleaned in 0.1 M sodium phosphate buffer (pH 7.8). Total RNA was extracted from each group of 20 pooled larvae with TRIzol reagent, treated with 5 U RQ1 DNase for 30 min at 37°C, cleaned up using a Qiagen RNeasy kit (Valencia, CA, USA), quantified by A_{260} and stored at −80°C.

RT-PCR Southern Analyses

RT-PCR Southern analyses were used to quantify the levels of the *CYP6B8*, *CYP6B9*, *CYP6B27*, and *CYP321A1* transcripts. To keep RT-PCR amplifications within their linear ranges, 50 μl RT-PCR reactions contained 100 ng total RNA, 0.5 μl RNasin (40 U/μl), 0.5 μl AMV reverse transcriptase (10 U/μl), 0.25 μl Taq polymerase (5 U/μl), 25 μl 2× RT buffer containing dNTPs, and 1 μl forward and reverse primer (10 μM) each. Gene-specific forward/reverse primer sets used in these reactions were (5′-TCTTGTGGACAGCATTAT TAGC-3′/5′-CAAGTCCGAATGGTAAGTACGC-3′) for CYP6B8, (5′-GATCTTT TATTTTCCGGCAGTG-3′/5′-CTTTCTCAATGACCACGTCTGTTC-3′) for CYP6B9, (5′-ATTTTTCCAACGGCGGTAAAG-3′/5′-CGGGAATTTTGTAATCTCTAGT-3′) for CYP6B27, (5′-TAGTGTGGAGGGTGACCAACTG-3′/5′-CGGACAACAAGCCAGTCG TAGGC-3′) for CYP321A1, and (5′-TGGGAYGAYATGGAGAAGATCTGG-3′/5′-TAGATGGGBACBGTGTGBGAGACA-3′ with Y (C/T), B (C/G/T)) for actin, which was used as a reference. RT-PCR reactions were initiated with first strand cDNA synthesis at 42°C for 50 min. After denaturation of the RNA-cDNA hybrid at 94°C for 5 min, 18 PCR amplification cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) were run and followed by a final extension for 5 min. Ten μl of concentrated (6×) loading dye were added to each reaction, and 20 μl of each sample were run on 1.0% agarose gel in 1×TBE buffer. After gel-blotting, the Hybond-N membranes (Amersham-Pharmacia Biosciences, Uppsala, Sweden) were prehybridized and then hybridized with P^{32}-labeled gene-specific probes at 42°C overnight in hybridization buffer containing 5× SSC, 5× Denhardt's solution, 50% formamide, 50 mM sodium phosphate (pH 7.0), and 0.5% SDS. Membranes were washed twice at low stringency in buffer containing 2×SSC, 0.1% SDS at 42°C each for 15 min and once at high stringency in buffer containing 0.2×SSC, 0.1% SDS at 62°C for 15 min. Membranes were autoradiographed and quantified by phosphoimager analysis using a Typhoon 8600 variable model imager (Amersham-Pharmacia Biotech). RT-PCR Southern analyses were repeated three times with RNA samples prepared from midguts of larvae in three independent biological replicates.

Statistical Analyses Data were evaluated by one-way analysis of variance (ANOVA) with treatment differences among means tested at $P=0.05$ using the Tukey *post hoc* test. All data for pupation rates are means from three replicates with 20 caterpillars/treatment. Because significant differences were found among replicates in pupal weights, this parameter was

analyzed independently for each replicate, and representative values from one replicate are reported.

Results

Indole-3-carbinol Dietary exposure of fourth and fifth *H. zea* instars to 1 mg/g I3C significantly reduced toxicity of 1 μg/g AFB1 to *H. zea*. The average weight gain, pupation rate and pupal weight of fifth instars on diets containing 1 mg/g I3C + 1 μg/g AFB1 increased by 108.0% ($P<0.001$), 69% ($P=0.002$), and 26.9% ($P<0.001$), respectively, compared with caterpillars on diets containing only 1 μg/g AFB1 (Fig. 1a–c). Pupation rates on diets containing 1 mg/g I3C + 1 μg/g AFB1 did not differ from rates on control diets (Fig. 1b).

Fourth instars on diets containing 1 mg/g I3C + 1 μg/g AFB1 gained 71.5% more weight ($P=0.036$) after 6 d of feeding compared with caterpillars on diets containing only 1 μg/g AFB1 (Fig. 1a). Whereas no fourth instars on diets containing 1 μg/g AFB1 succeeded in pupating, addition of 1 mg/g I3C to the diet increased pupation rates to 40% and pupal weights to 261.3 mg ($P<0.001$) (Fig. 1b, c).

Coumarin Fifth instars consuming diets containing 1 mg/g COU + 1 μg/g AFB1 displayed weight gain, pupation rate, and pupal weight increases of 139.1% ($P<0.001$), 40.5% ($P=0.035$), and 23.6% ($P=0.002$), respectively, compared with caterpillars receiving only 1 μg/g AFB1 (Fig. 2a–c). Pupation rates and weight gain on diet containing 1 mg/g COU + 1 μg/g AFB1 were equivalent to those on control diet (Fig. 2a, b). Pupation rate (63.3%) ($P<0.001$) and pupal weights (265.1 mg) ($P<0.001$) of fourth instars on diets containing 1 mg/g COU + 1 μg/g AFB1 were significantly higher than those of *H. zea* on diets containing AFB1 alone (5.0% pupation rates and 164 mg pupal weight) (Fig. 2b, c). Caterpillars on diet containing COU + AFB1 increased in size by 216.1% after 6 d of treatments ($P<0.001$) (Fig. 2a).

Xanthotoxin Caterpillars simultaneously treated with XAN and AFB1 displayed substantially greater tolerance to AFB1 compared with those exposed only to AFB1. Weight gain of fifth instars on diets containing 0.5 mg/g XAN + 1 μg/g AFB1 after 3 d of treatment increased by 135.2% ($P=0.0017$) (Fig. 3a), and pupal weights increased by 29.3% ($P<0.001$) (Fig. 3c) compared to *H. zea* on diets containing AFB1 alone. Even more strikingly, the weight gain of fourth instars after 6 d on a diet containing 0.5 mg/g XAN + 1 μg/g AFB1 increased by 700% ($P<0.001$) (Fig. 3a) compared to caterpillars on diets containing only AFB1. Whereas no fourth instars on diets containing 1 μg/g AFB1 succeeded in pupating, addition of 0.25 mg/g XAN to the diet increased pupation rates to 88.3% and pupal weights to 279 mg ($P<0.001$) (Fig. 3b, c). Pupation rates in the presence of XAN were, in fact, not significantly different from pupation rates of larvae on control diets ($P=0.057$).

Insecticides Five hours after exposure to diazinon, fifth instars consuming control diets experienced 68.8% mortality. In contrast, caterpillars fed for 48 hr on diets containing COU, I3C, or XAN experienced mortality rates of 18.8% ($P<0.001$), 31.3% ($P=0.005$), and 36.3% ($P=0.013$), respectively, after diazinon treatment (Fig. 4). Similarly, where fifth instars consuming control diets experienced 68.7% mortality 5 hr after carbaryl exposure,

H. zea consuming diets containing COU, I3C, and XAN experienced mortality rates of 25.0% (P=0.004), 38.8% (P=0.048), and 37.5% (P=0.038), respectively, after carbaryl exposure (Fig. 4).

Induction of P450 Transcripts To determine whether COU, I3C, and XAN enhance the detoxification of AFB1 by inducing transcription of P450 genes in *H. zea*, RNA samples were analyzed from fifth instars fed for 48 hr on diets supplemented with each of the three plant allelochemicals. All three chemicals induced accumulation of *CYP321A1* transcripts over basal levels present in fifth instar control midguts (Fig. 5). *CYP321A1* transcripts accumulated approximately to 10.6-fold higher levels in response to 0.5 mg/g XAN, 8.3-fold higher levels in response to 1 mg/g I3C, and 20.7-fold higher levels in response to

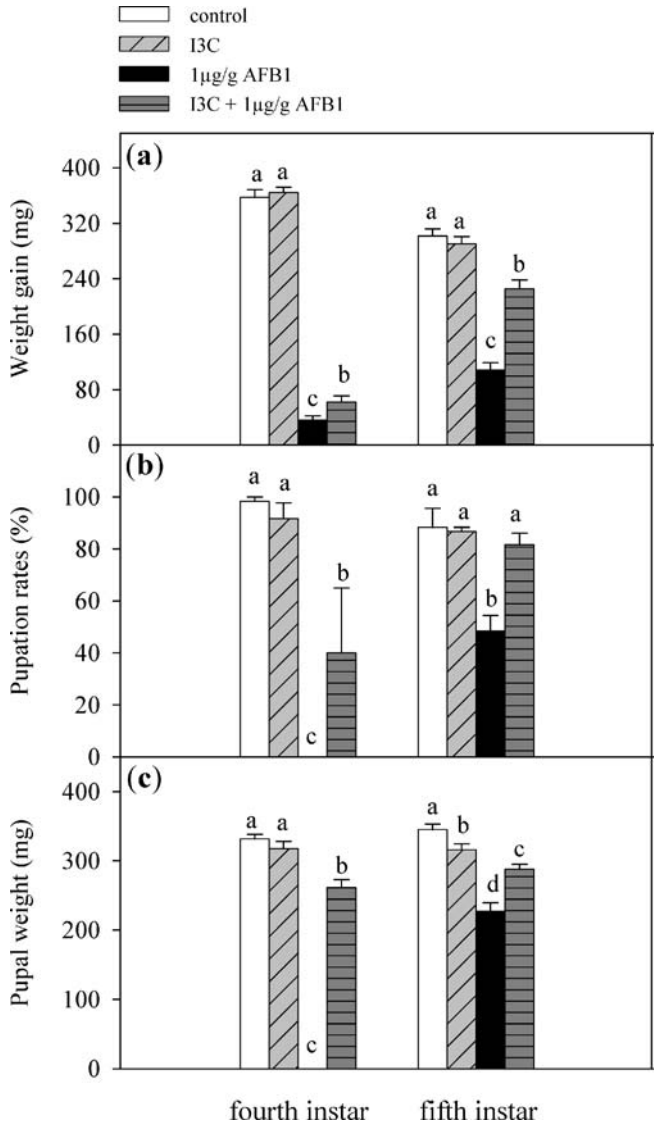


Fig. 1 Effects of I3C on growth and pupation of *H. zea* fourth and fifth instars exposed to 1 μg/g AFB1. Values shown for pupation rate are means+standard error from three replicates with 20 caterpillars/treatment. Values for weight gain and pupal weight are representative of one of the three series of replicates. Significant differences (P<0.05 using Tukey *post hoc* test) among treatments in a group are indicated by different letters above the bars

1 mg/g COU. Comparison among transcripts indicated that the induction responses were gene-specific. *CYP6B8* transcripts were induced approximately 2.6-fold by 1 mg/g COU, 2.6-fold by 0.5 mg/g XAN, and not at all by I3C (Fig. 5). Neither *CYP6B8* nor *CYP321A1* was upregulated in the presence of AFB1 alone. The other two P450 transcripts analyzed, *CYP6B9* and *CYP6B27*, were not induced significantly by any of these compounds.

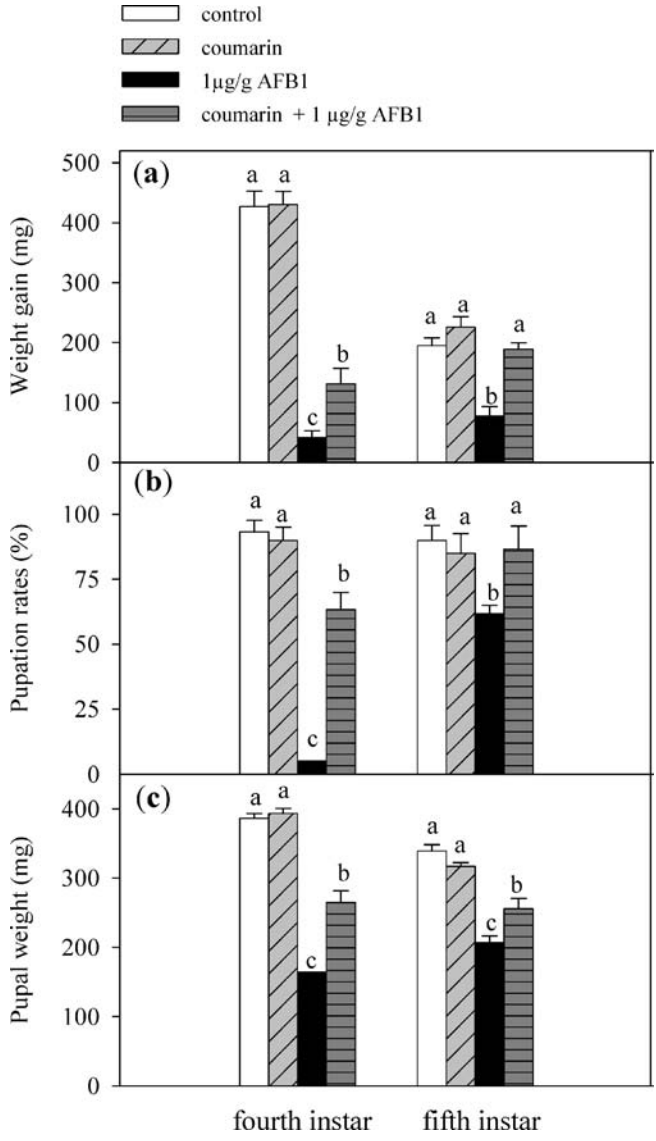


Fig. 2 Effects of COU on growth and pupation of *H. zea* fourth and fifth instars exposed to 1 μg/g AFB1. Values shown for pupation rate are means+standard error from three replicates with 20 caterpillars/treatment. Values for weight gain and pupal weight are representative of one of the three series of replicates. Significant differences ($P<0.05$ using Tukey *post hoc* test) among treatments in a group are indicated by different letters above the bars

Discussion

Plant allelochemicals have been demonstrated or implicated in chemoprotection in many vertebrate systems (Bailey et al., 1987; Grubbs et al., 1995; Takahashi et al., 1995a,b; Goeger et al., 1998, 1999; Manson et al., 1998; Xu et al., 2001; Stoner et al., 2002). These effects are generally attributed to the ability of these compounds to induce detoxification enzymes, mainly P450s. While a large body of evidence has shown that plant allelochemicals induce P450s in insects, rarely have the experiments been carried out to demonstrate a correlation between the induction of P450s and the enhancement of an insect's tolerance to toxins. Feeding on a broad range of plant species, including many of

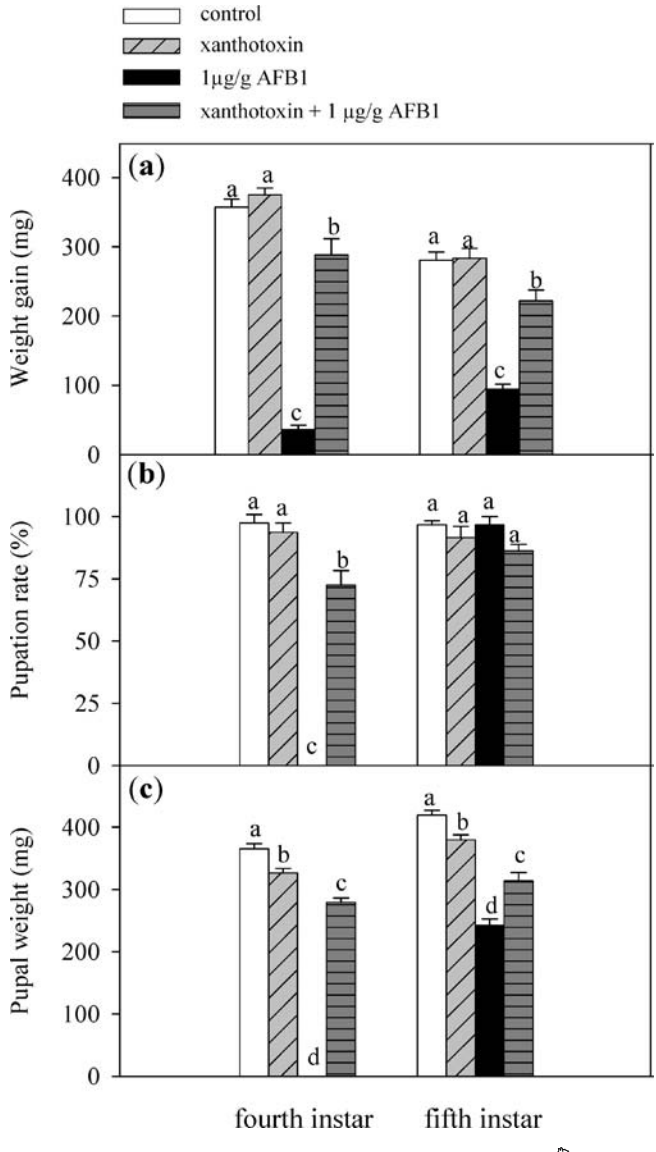


Fig. 3 Effects of XAN on growth and pupation of *H. zea* fourth and fifth instars exposed to 1 μg/g AFB1. Values shown for pupation rate are means+standard error from three replicates with 20 caterpillars/treatment. Values for weight gain and pupal weight are representative of one of the three series of replicates. Significant differences ($P<0.05$ using Tukey *post hoc* test) among treatments in a group are indicated by different letters above the bars

agricultural importance that are susceptible to infection by *Aspergillus*, assures that corn earworms are exposed to mycotoxins and/or insecticides concurrent with the many allelochemicals in their host plants. The corn earworm, therefore, represents a good model to determine whether there is a correlation between P450 induction by plant allelochemicals and the effects of these chemicals on the toxicity of mycotoxins and insecticides to insects.

The frequency with which herbivorous insects encounter a specific allelochemical depends on many factors including the distribution of allelochemicals throughout the host range and the frequency with which a host plant is used. Of the three allelochemicals examined, COU is widely distributed in host plants and frequently encountered by *H. zea*, I3C is derived from glucosinolates in cruciferous plants and occasionally encountered (Chevolleau et al., 1997), and XAN is found in several plant families including Apiaceae and Rutaceae (Berenbaum, 1991) and rarely encountered. Our data show that all three allelochemicals substantially reduced the toxicity of AFB1, diazinon, and carbaryl to *H. zea*. In parallel with their effects in reducing toxicity of AFB1, diazinon, and carbaryl to *H. zea*, these allelochemicals selectively induced the transcription of midgut P450s in *H. zea* larvae. Of the four P450s examined, CYP6B8 transcripts were induced 2.6-fold by both COU and XAN, and CYP321A1 transcripts were induced at a much higher level by all three allelochemicals (20.7-fold by COU, 8.3-fold by I3C, and 10.6-fold by XAN). Both P450s have been shown to metabolize many different types of toxins including insecticides in vitro (Li et al., 2004; Sasabe et al., 2004). These results indicate that induction of toxin-

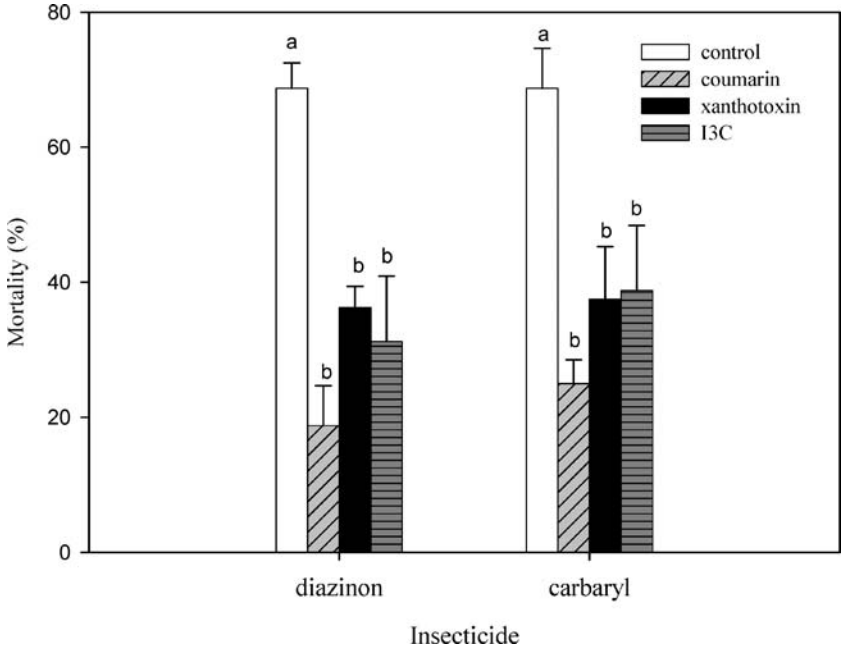


Fig. 4 Allelochemical-induced insecticide resistance. After exposure of newly molted fifth instars to diets containing 0.2% DMSO (control) plus 1 mg/g COU, 1 mg/g I3C or 0.5 mg/g XAN for 48 hr, the mortality rates of *H. zea* were monitored after 5 hr of treatment with 300 μg diazinon/caterpillar or 100 μg carbaryl/caterpillar. Values are means+standard errors from four replicates with 20 caterpillars/replicate. Significant differences ($P<0.05$ using Tukey *post hoc* test) among treatments in a group are indicated by different letters above the bars

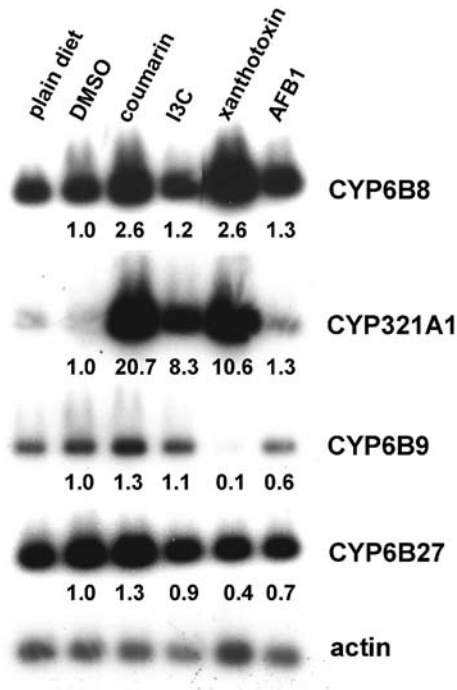


Fig. 5 P450 transcript induction by plant allelochemicals. Total RNA pooled from 20 midguts of newly molted fifth instar *H. zea* feeding for 48 hr on diet containing plant allelochemicals. Control, unamended diet; DMSO, diet containing 0.2% DMSO (used as a solvent to dissolve the tested allelochemicals); coumarin, diet containing 1 mg/g COU; I3C, diet containing 1 mg/g I3C; xanthotoxin, diet containing 0.5 mg/g XAN; AFB1, diet containing 1 μg/g AFB1. The induction level for each was calculated by normalizing the signal of each gene against actin and then using the DMSO control as a reference point

detoxifying P450s in insects significantly enhances this insect's tolerance to toxins. The responses to XAN are especially interesting. Despite the fact that only slightly higher concentrations of XAN than tested here are toxic to *H. zea* (Berenbaum and Neal, 1985) and that this furanocoumarin is only rarely encountered by this species, it is extremely effective at reducing the toxicity of AFB1 and insecticides to *H. zea* and inducing *CYP6B8* and *CYP321A1* transcript accumulation.

Synergistic interactions arising from inhibition of detoxification enzymes by cooccurring plant allelochemicals have been exploited in the development of commercial synergists for pesticides designed to circumvent metabolic resistance. Indeed, sesamin, a methylenedioxyphenyl compound found in *Chrysanthemum* (=*Tanacetum*) *cinerariaefolium*, the pyrethrum plant, is among the oldest of commercial pyrethroid synergists (Whittaker and Feeny, 1971). By the same token, understanding the sort of antagonistic interactions among plant constituents demonstrated here, according to which inducers of detoxification enzymes reduce toxicity of cooccurring allelochemicals, can provide insights into optimizing chemically based pest management programs, potentially providing mechanistic explanations for the differential efficacy of insecticides on different crop species or varieties (e.g., Alverson and Gorsuch, 1982; Kennedy, 1984; Abro and Wright, 1989).

Acknowledgments This research was supported by U.S. Department of Agriculture grant 01-35302-10884 to M.A.S. and M.R.B., National Institutes of Health grant GM071826 to M.A.S. and Natural Science Foundation of China (30370246, 30670331) and Guangdong (Group Project 039254, 04105977), National 973 project of China (2006CB100200), Guangdong Science & Technology Plan Program (2004B20501010) and Program for New Century Excellent Talents in University (NCET-04-0830) to R.S.Z. We thank Dr. Arthur Zangerl for technical assistance and Allen Lawrence and Henan Zeng for help with insect rearing. R. S. Zeng and Z. Wen contributed equally to the work.

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J Chem Ecol (2007) 33:463–475
DOI 10.1007/s10886-006-9239-0

Temperature as a Modifier of Plant–Herbivore Interaction

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Received: 14 August 2006 / Accepted: 5 December 2006 /
Published online: 1 February 2007
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Abstract Temperature directly affects the growth, survival, and development rates of poikilothermic insect herbivores; it may also have an important indirect impact, via the activities of plant defensive enzymes. The effects of wounding birch leaves and temperature on the growth and development rates of a Lepidopteran moth, *Epirrita autumnata*, were studied. We also examined the activities of a mountain birch (*Betula pubescesns* spp. *czerepanovii*) defensive enzymes, specifically the polyphenoloxidases (PPOs), in relation to temperature and wounding. The optimal temperature for early instars in terms of survival and developmental rates was between +15 and 20°C. Wounding treatment had different effects on birch PPO activity depending on the temperature: at +12°C, wounding decreased the activity, suggesting induced amelioration at that temperature, whereas at +25°C, wounding increased the activity, suggesting induced resistance. However, larval growth was retarded slightly, but significantly, on the leaves of wounded twigs at both temperatures. Both PPO activity and larval growth rates were affected within 12 h, indicating the existence of a transcription- and translation-independent defense system in birch leaves. We suggest that underlying the increase in PPO activity and the decrease in larval growth rate may be H_2O_2, which has been shown to accumulate in response to wounding. Our results also provide a possible biological mechanism for the hypothesis that low temperatures promote the success of *E. autumnata* and other Lepidopteran larvae via decreased defensive enzyme activities of host plants at lower temperatures.

Keywords Temperature · Plant–herbivore interaction · *Epirrita autumnata* · Mountain birch · Polyphenol oxidase

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Introduction

Temperature is one of the fundamental abiotic factors that lay down the boundaries for the ranges of animals and plants. Temperature affects enzyme activities, growth, development rates, and further population dynamics of plant-eating animals. Food quality is a bottom-up factor that varies spatially and temporally; it is affected by abiotic factors, such as ambient temperature, and by biotic factors, such as wounding by herbivores (Scriber and Slansky, 1981). Plants are not silent victims; they are defended against herbivores by multiple strategies, and an herbivore attack triggers complex and delicate signaling pathways that lead to an activated defense (Lamb and Dixon, 1997).

A number of studies have shown that foliar phenoloxidase activities have a negative impact on the performance of several species of insect herbivores (e.g., Felton et al., 1989; Bi and Felton, 1995; Tscharntke et al., 2001). Polyphenol oxidases (PPOs) are ubiquitous enzymes found in both plants and animals. PPOs are located in plastids (Vaughn and Duke, 1984), apart from their vacuolar phenolic substrates, but the rupture of cell compartments brings the enzymes together with their substrates. PPOs use several plant phenolics as substrates, converting them to highly reactive quinones that attach covalently to the electron-rich moieties of multiple compounds, including amino acids and proteins (Felton et al., 1989). Precipitation of amino acids, especially electron-rich essential amino acids, is suggested to decrease the nutritive value of plant tissues for herbivores, reducing, in turn, the growth rate of juvenile insects (Felton et al., 1989).

Wounding and herbivory also trigger production of reactive oxygen species such as H_2O_2 (Bi and Felton, 1995; Orozco-Cárdenas et al., 2001; Ruuhola and Yang, 2006), which have direct defensive roles: for instance, acting as a feeding deterrent (Lamb and Dixon, 1997) or causing oxidative damage in the insect midgut (Bi and Felton, 1995). In addition, H_2O_2 is also a substrate of peroxidases (PODs) and mediates distress signaling in plants (Lamb and Dixon, 1997; Alvarez et al., 1998). Like PPOs, PODs use plant phenolics as substrates, converting them to quinones, but they have multiple other roles in addition to defense, including lignification, suberization, and the cross-linking of cell walls proteins (Hiraga et al., 2001). Overall, the role of defensive enzymes has largely been ignored in ecological studies focusing on the roles of secondary compounds in plant–herbivory interactions.

Cool springtime temperatures have often been associated with the outbreaks of several species of forest Lepidoptera in the northern hemisphere (Myers, 1998), including *Epirrita autumnata* (Lepidoptera: Geometridae) (Niemelä, 1980; see also Haukioja et al., 1988). Weather conditions have been suggested to act as a Moran effect, synchronizing the fluctuation of Lepidopteran species over wide geographic areas (Myers, 1998). However, the biological mechanisms that explain the link between cool temperature and Lepidopteran outbreaks are not known.

Some insect species, such as *E. autumnata*, are adapted to a wide temperature range. The geometrid *E. autumnata* is common throughout most of the Holoarctic region and shows regular large-scale outbreaks in mountain birch forests of the northernmost mountainous area (Tenow, 1972). As temperature is a critical determinant for enzyme activities, we predicted that mountain birch foliar oxidase activities might be considerably hindered at low temperatures. We undertook a study to examine the effect of temperature on foliar enzyme activities and on the growth of young instar *E. autumnata* and their interaction.

Methods and Materials

Experimental Organisms Experiments were conducted in the laboratory of the Kevo Subarctic Research Institute of the University of Turku (69°45′ N, 27°00′ E) in summer 2003. Mountain birch [*Betula pubescens* spp. czerepanovii (Orlova) Hämet-Ahti] forms uniform stands at the arctic tree line in northern Fennoscandia. Mountain birch harbors over 50 species of folivorous insects (Koponen, 1973), *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae) being the most deleterious species (Tenow, 1972, 1975; Kallio and Lehtonen, 1973).

E. autumnata larvae are highly polyphagous, but in the subarctic area the main host is the mountain birch, due apparently to its abundance. Larvae hatch in spring at the time of mountain birch leaf-flush (Haukioja et al., 1988; Kaitaniemi and Ruohomäki, 1999); the successful synchronization of hatching with bud burst is essential for larval development (Ayres and MacLean, 1987a; Tammaru, 1998). Low-altitude sites, such as river valleys, regularly experience low winter temperatures, killing the eggs. Outbreaks usually occur at high-altitude sites (Tenow, 1972, 1975; Kallio and Lehtonen, 1973; Virtanen et al., 1998), but cannot occur after a cold winter (Ruohomäki et al., 2000).

Experimental Design We used two temperatures: a constant +12°C, corresponding to the mean temperature of *E. autumnata* larval period, which has been used regularly in the bioassay of *E. autumnata* and other birch herbivores (e.g., Kause et al., 1999; Riipi et al., 2002; Lempa et al., 2004; Ruusila et al., 2005; Kapari et al., 2006), and +25°C, corresponding to the temperature of a warm summer day. These two temperatures were originally selected to test methodological aspects: how do regularly used rearing temperatures of *E. autumnata* (+12 and +25°C) affect the results of bioassays? In other words, we did not want to test only direct effects of rearing temperatures, but also indirect effects via temperature-modified changes in leaf enzyme activities. In addition, this study helps in the evaluation of ecological aspects of temperature in nature, as a high variation between night and day temperatures is characteristic for subarctic climate, but warm summer days occur regularly. For example, at end of June 2003, Kevo was the warmest region in Finland.

We selected, haphazardly, 10 mountain birches growing on the upper part of the Puksala fell. In early June, four branches were cut from each tree and brought to the laboratory. Isolated plant organs are used routinely in physiological studies, and isolation does not normally prevent local enzyme induction (e.g., Constabel and Ryan, 1998). Four twigs (length ca 1 m) from each tree were randomly allocated to four temperature–treatment combinations: +25 control, +25°C larval treatment, +12°C control, and +12°C larval treatment (Table 1). All the twigs were placed into holders, and the freshly cut ends were put into water. The experiment was started by releasing 10 larvae onto the larval twigs (10 larvae per treated twig). The larvae were allowed to feed freely on the larval twigs for 12 h, after which sampling for the enzyme analyses and for the RGR-experiment was conducted (Table 1).

Bioassay with Larvae *E. autumnata* larvae originating from the crossing of previous-season moths collected both from the Kevo area and an outbreak area in Norway, were reared individually in plastic 48-ml vials under a natural temperature regime until they molted to the third instar. Larvae were fed with mountain birch leaves every second or third day; thus, they had fresh food and did not suffer from a shortage of food. After molting to the third instar, larvae were placed at +1°C without food. When all had molted, new fresh leaves were provided to larvae for pre-feeding 12 h before the experiment (+12°C).

Table 1 The scheme of experimental procedure of a short-term temperature trial

Ten mountain birch trees, four twigs from each tree, pre-incubation 12 h[a]					
Two twigs at +12°C			Two twigs at +25°C		
A control twig	A larval twig		A control twig	A larval twig[a]	
Twenty-four-hour bioassay with third instars					
+12°C (150 larvae; 50 larvae/treatment)			+25°C (150 larvae; 50 larvae/treatment)		
Unwounded shoot from the control twig	Unwounded[b] shoot from the larval twig	Wounded[b] shoot from the larval twig	Unwounded shoot from the control twig	Unwounded[b] shoot from the larval twig	Wounded[b] shoot from the larval twig
Enzymatic assays[c]					
+12°C			+25°C		
Unwounded shoots from the control twig	Unwounded shoots from the larval twig		Unwounded shoots from the control twig	Unwounded shoots from the larval twig	

[a] Ten *E. autumnata* larvae were released on each larval twig and were allowed to feed freely for 12 h. Larvae were at their third instar and reared in lab conditions.

[b] This data (temperatures were kept separately) was combined in the statistical procedure.

[c] Enzyme samples were collected at the same time when the growth trial was started.

We used 10 broods and 30 larvae per brood, divided between 2 temperatures and 3 treatments per temperature (50 larvae per treatment, total 300 larvae; Table 1). For the bioassay, 5 short shoots from each control twig were cut randomly for 5 larvae, and 5 wounded and 5 unwounded short shoots from the larval twigs were selected for 10 larvae (each control twig was repeated 5 times; larval twigs 10 times). The cut ends of the short shoots were placed into a small cup of water to prevent the leaves from drying out during the experiment. Shoots and larvae were placed individually into a 100-ml jar after determination of the initial larval weight. Larvae on short shoots collected from twigs incubated at +12°C were reared at this temperature, whereas larvae on short shoots incubated at +25°C were correspondingly reared at that temperature (see Table 1). Larvae were reared at both temperatures for 24 h, after which final weights were determined.

Protein Extraction and Enzymatic Assays Twelve hours after release of larvae onto the larval twigs, leaf samples were collected for enzyme analyses (Table 1). Undamaged leaves from both control and larval twigs were collected and immediately frozen in liquid nitrogen, and stored at −80°C before the analyses. Enzyme extractions were conducted as described in Ruuhola and Yang (2006). We used the 1-mM serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Sigma) in the extraction buffer to prevent proteolysis and activation of latent PPO during the isolation process.

Enzyme activities were measured with a spectrophotometer coupled to an external water bath to control the reaction temperature, using methods described in more detail in Ruuhola and Yang (2006). Enzyme activities were measured at the corresponding experimental temperatures: +12 and +25°C. Catechol oxidase activity of PPOs (EC 1.10.2.2) was measured at pH 5.8, with 80 mM catechol as a substrate at wavelength of 420 nm; combined PPO/POD activity was measured similarly, but with 6.5 nM H_2O_2 as a co-substrate. The protein assay method of BioRad was used to measure protein contents of enzyme samples. All measurements were conducted in triplicate.

Temperature Optima for Birch Oxidases Temperature optima for foliar oxidase activities were tested by measuring enzyme activities at temperatures raging from +6 to +72°C for PPO and from +6 to +58°C for combined PPO/POD, which was measured in the presence of exogenous H_2O_2. Three enzyme samples were haphazardly chosen from both the control and larval treatments for determination of temperature optima. The enzymes themselves were kept on ice; substrates and buffers were kept at measurement temperature, and a circulating water bath was set to the same temperature. All measurements were conducted in triplicate; the reaction time was 3 min.

Temperature Optima for Development Rate of Moth Larvae For the range finding of optimal temperature for the development rate of early instars, *E. autumnata* larvae (50 larvae per temperature) were reared on an artificial diet at five different temperatures ranging from +5 to +25°C. The diet was based on the recipe of Goujet and Guilbort (in Singh, 1985), modified as in Ruuhola et al. (2001). This time, however, we used powdered mountain birch leaves instead of powdered *Salix phylicifolia* leaves. Newly hatched first instars were transferred individually onto a piece of the artificial diet in sterile petri dishes, diameter 6 cm. Dishes were enclosed with parafilm to prevent the escape of small insects. The piece of food was changed every other day.

Statistical Analyses We analyzed data with generalized mixed linear models (procedure MIXED in the SAS statistical software, release 8.2). The dependent variable was the growth of *E. autumnata* (final weight−initial weight within 24 h). As initial weight is the major determinant for the final weight, it was set as a covariate in the analysis. Fixed effects in the analyses were temperature (+12 and +25°C), defoliation treatment (control or damaged twigs), initial weight (as a covariate), and treatment×initial weight interactions. To simplify the model, we calculated the mean for two larvae grown on damaged and adjacent undamaged leaves (the mean for larvae belonging to same brood grown on the same twig; one on the undamaged adjacent leaf, the other on the damaged leaf). These two treatments did not differ statistically in larval growth rates. Experimental tree was handled as a subject and larval brood as a random effect. To compute the denominator degrees of freedom for the tests of fixed effects, we applied the method of *Satterthwaite*. The effect of temperature on the duration of first and second *E. autumnata* instars (that were reared on the artificial diet at five different temperatures) were tested with a nonparametric *Kruskal–Wallis's* test (procedure *NPAR1WAY* in the SAS statistical software, release 8.2)

Enzyme activities were also tested with generalized mixed linear models (procedure MIXED in the SAS statistical software, release 8.2). Fixed effects were temperature and defoliation treatment and their interaction; tree was assigned as a subject and a random effect. We used contrasts to clarify the relationship between temperature and treatment in the PPO activities that showed a significant treatment×temperature interaction.

Results

Larval Growth in Relation to Temperature and Foliar Wounding The growth rates of *E. autumnata* third instars were markedly affected by growth temperature: the growth rate was over twice as fast at +25°C as at +12°C (Fig. 1). In addition, growth was slightly, but significantly, slower on leaves of larval twigs than on those of control twigs (Table 2,

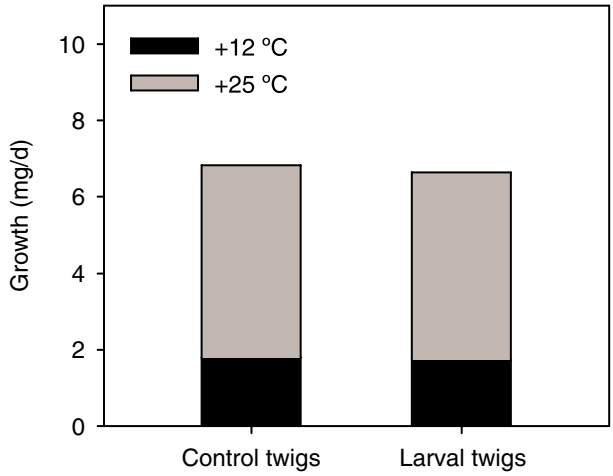


Fig. 1 Growth rate (final weight−initial weight within 24 h) of third instar *Epirrita autumnata* at two temperatures on the leaves of control and wounded twigs of mountain birch

Fig. 1). Although larvae were randomly allocated to treatments, the treatment×initial weight interaction was also significant (Table 2). Larvae with different initial weight probably responded differentially to the treatments.

Temperature affected development time of larvae (duration of the first instar *Chi-square*=147.8, $P<0.001$; duration of the second instar *Chi-square*=101.7, $P<0.001$). The optimum temperature for larval growth was +25°C; at this temperature, development from first to third instar lasted an average of 6.4 days (Fig. 2). At +20°C, development time was 1 day longer; at +15°C, development was 3.5 days longer than at +25°C. After that, development time increased rapidly; at +10°C, it was twice as long as at +15°C. At +5°C, the duration of first instar lasted as long as 25 days; we did not measure the development time to the third instar. At temperatures from +15 to +25°C, the duration of the second instar was shorter than that of the first instar, while at +10°C, the duration of the first instar was shorter than that of the second.

Although, the development rate of *E. autumnata* larvae was fastest at +25°C, larval mortality also increased markedly at this temperature (Fig. 2). Larval mortality was also high at the other end of the temperature range, i.e., at +5°C. Mortality rate was lowest between +10 and 20°C. According to survival and development rates, the optimum temperature for young instars was between +15 and 20°C.

Table 2 Mixed model results of larval growth at two different temperatures and treatments (SAS Institute)

Test of fixed effects

Effect	Num df	Den df	*F* value	*Pr>F*
Temperature	1	143	1,008	<0.001
Treatment	1	191	9.57	0.002
Initial weight	1	192	25.9	<0.001
Treatment×initial weight	1	191	9.25	0.003

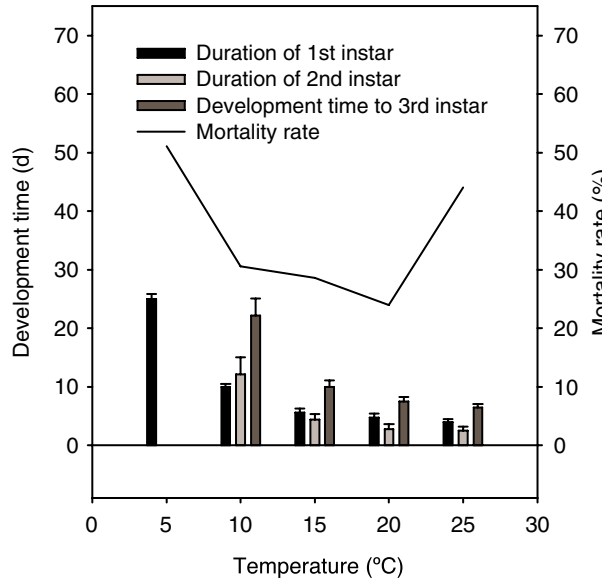


Fig. 2 Development times and mortality rates of early instar *Epirrita autumnata* at different temperatures. The larvae were grown on an artificial diet to avoid the effect of tree phenology. *Bars* represent SE

Foliar Enzyme Activity in Relation to Temperature and Wounding We found significant temperature×wounding interaction in the activity of foliar PPOs (Table 3, Fig. 3A and B). Wounded twigs at +25°C had higher enzyme activities than wounded twigs at +12°C (contrast between wounding treatments at +12 and +25°C: specific PPO activity: $F_{1,27}$= 12.67, P=0.001 and PPO activity per gram leaf material (FW): $F_{1,27}$=15.25, P=0.001), whereas control treatments did not differ (contrast between control treatments in +12 and +25°C: specific PPO activity: $F_{1,27}$=0.54, P=0.467 and PPO activity g leaf fw^{-1}: $F_{1,27}$= 0.54, P=0.469). Interestingly, at +12°C, leaf damage decreased PPO activities, whereas at +25°C, leaf damage increased PPO activities in the adjacent undamaged leaves (Fig. 3A and B). Temperature also markedly affected the activity of foliar PPOs; both specific activity and activity per gram leaf material (FW) were significantly higher at +25°C than at +12°C (Table 3, Fig. 3A and B). The wounding treatment as such had no effect on activity (Table 3). Temperature, but not wounding, affected combined PPO/POD activities (Table 3, Fig. 3C and D).

POD activities were not affected by temperature or wounding treatment (Table 3, Fig. 3E and F). Interestingly, POD activities showed an opposite tendency (but nonsignificant) than PPO activities; activity was higher in leaves of larval branches than control leaves at +12°C, but lower at +25°C. In contrast, the activity of antioxidative catalase was affected by temperature; the activity being higher at +25°C than at +12°C, and the wounding treatment had a marginally increasing effect on catalase activity (Table 3, Fig. 3G and H).

Foliar PPO showed a surprisingly high temperature optimum: activity increased until +55°C, after which it collapsed (Fig. 4A). Our preliminary results suggest that birch PPOs are actually heat-labile enzymes, as activity declined rapidly when the enzyme itself was incubated at +55°C (data not shown). The range finding of temperature optima for combined PPO/POD activity was measured independently, using catechol and H_2O_2 as substrate and co-substrate respectively. This combined activity was also surprisingly high at cold temperatures, but started to decline after +25°C (Fig. 4B).

Discussion

Growth rate of third-instar *E. autumnata* was greatly affected by growth temperature; at +25°C, larvae grew twice as fast as at +12°C. Larvae also grew more slowly on leaves of larval than of control twigs. Both rapidly induced resistance (e.g., Haukioja and Hanhimäki, 1985; Kaitaniemi and Ruohomäki, 2001) and delayed induced resistance (e.g., Haukioja et al., 1985; Ruohomäki et al., 1992, 1996) are well-established phenomena in mountain birch, although induced amelioration has also been reported in mountain birch as a response to herbivory (Lempa et al., 2004). Our study, however, is the first to find induced resistance of mountain birch as an immediate reaction occurring within 12 h, suggesting the existence of a rapid transcription- and translation-independent defense mechanism.

In the range-finding experiment for the temperature optimum, we found that development rate from first to third instar was also fastest at +25°C, although larval survival rate was poor at that temperature. The optimal growth and survival rates of early instars were between +15 and +20°C. Larvae, however, were able to develop at temperatures as low as +5°C, even though development from first to second instar took almost a month. The development rate of autumnal moth larvae is known to be highly temperature-dependent; the length of *E. autumnata* larval stage depends on the ambient

Table 3 Mixed model results (SAS institute) of the effects of temperature, treatment, and their interaction on the mountain birch foliar oxidases

Test of fixed effects

Depended variable		Temperature	Treatment	Temp×treat
PPO (U×mg protein^{-1})	Den df	**27**	27	*27*
	F	**9.23**	0.02	*3.98*
	P	**0.005**	0.917	*0.056*
PPO (U×g leaf fw^{-1})	Den df	**27**	27	**27**
	F	**10.76**	0.10	**5.03**
	P	**0.003**	0.754	**0.033**
PPO/POD (U×mg protein^{-1})	Den df	**26.3**	26.3	26.3
	F	**6.04**	0.11	0.20
	P	**0.021**	0.746	0.662
PPO/POD (U×g leaf fw^{-1})	Den df	**26.1**	26.1	26.1
	F	**5.61**	0.06	0.01
	P	**0.026**	0.815	0.915
POD (U×mg protein^{-1})	Den df	27	27	27
	F	1.52	0.00	1.84
	P	0.229	0.959	0.183
POD (U×g leaf fw^{-1})	Den df	27	27	27
	F	1.35	0.30	1.92
	P	0.256	0.585	0.177
Catalase (U×mg protein^{-1})	Den df	**27**	27	27
	F	**13.13**	0.86	0.26
	P	**0.001**	0.361	0.614
Catalase (U×g leaf fw^{-1})	Den df	**26.2**	*26.2*	26.2
	F	**4.54**	*3.00*	0.41
	P	**0.043**	*0.095*	0.528

Statistically significant cases are in bold ($P \leq 0.05$; $P \leq 0.01$; $P \leq 0.001$) and close to significant cases are in italics ($P \leq 0.1$).

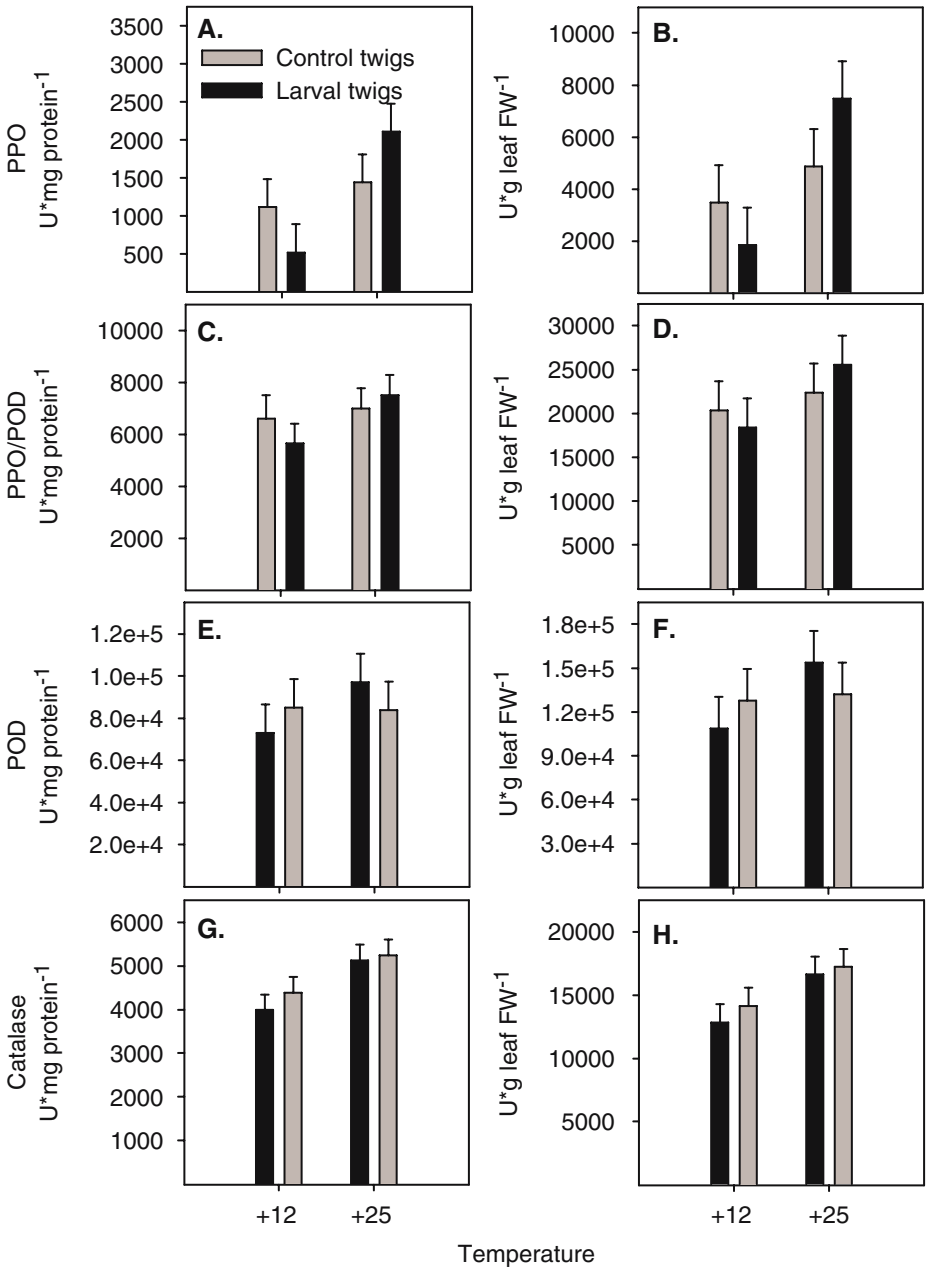


Fig. 3 Effect of larval wounding on the PPO (**A**–**B**), PPO/POD (**C**–**D**), POD (**E**–**F**), and Catalase (**G**–**H**) activities in the mountain birch leaves at two temperatures. *Bars* represent SE; $N=10$

temperature, varying from 2 weeks to almost 2 months (Ruohomäki et al., 2000). In other words, warm summer temperatures seem to be advantageous for the autumnal moth, enabling a rapid development rate and a short larval stage. At temperatures above or below the optimum, insect performance is expected to decrease (Scriber and Slansky, 1981); in

other words, insect growth rate is not usually linear in relation to temperature. Ayres and MacLean (1987b) found that low temperatures retarded the development of molting larvae more than growing larvae; thus, ambient temperature is apparently critical for larval molting from one instar to another, and may partly explain the nonlinearity of development rates in relation to temperature.

Warm early season temperatures may also have adverse consequences, in spite of their evident growth-promoting effects. Virtanen and Neuvonen (1999) reported that *E. autumnata* larval survival and females' fecundity were lower and larval parasitism higher at warmer than at lower temperatures. The 3-week delay in larval hatching, when the weather was cold and leaf development slow, had no significant effect on the survival of the first instar or on larval weight gain (in Haukioja, 1980). In addition, larvae that had hatched too early also remained active longer at cool temperatures, giving them a better chance to find food and survive (Haukioja et al., 1988). In our study too, the mortality of young instars increased at +25°C; the first instars, on the other hand, were able to survive at +5°C, feeding minimally, for almost a month—some larvae even succeeded in molting to the second instar.

Temperature and food quality interact to determine the suitability of plants for herbivores. For example, the effects of allelochemicals from Solanaceae species on the relative growth rates of *Manducta sexta*, *Helicorpa zea*, and *Spodoptera exigua* depended greatly on thermal regime, from a growth-promoting effect to a negative, growth-retarding one (Stamp and Yang, 1996). Also, temperature interacts with food quality in the interaction between mountain birch and autumnal moth: the growth rate of fifth instars was more affected by leaf age at +24°C than at +6°C (Ayres and MacLean, 1987a).

The birch defensive enzyme PPO showed interesting features. At +12°C, foliar PPO activity was reduced in leaves of larval twigs. At +25°C, in contrast, wounding increased PPO activity, suggesting an induced susceptibility of birches at low temperatures and an induced resistance at higher temperatures. Moth larvae used in the wounding treatment did not feed more than 12 h on the branches at both treatment temperatures, indicating that PPO activity responds very quickly to the presence of feeding larvae. In our earlier study (Ruuhola and Yang, 2006), PPO activity was induced systemically within 24 h in branches distinct from the wounded sites, confirming the quick induction of birch PPOs.

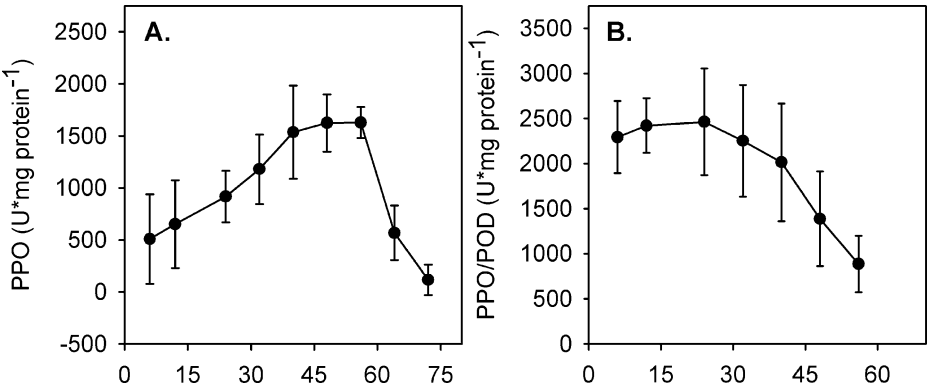


Fig. 4 Optimal temperatures for PPO (**A**) and PPO/POD (**B**) activities. Pure PPO activity was measured with catechol as a substrate and combined PPO/POD activity with catechol as a substrate and H_2O_2 as a co-substrate. *Bars* represent SE; $N=6$

The second intriguing feature of birch PPO was the high temperature optimum: the highest activities were found between +25 and 55°C. Heat-shock induction of PPOs has been reported for other plant species (Mathew and Parpia, 1971; Lee et al., 1991; Doğan et al., 2005) and could be due to the release of latent PPO (Lee et al., 1991) or a conformational change in a polypeptide (Mathew and Parpia, 1971). Doan et al. (2004) found that PPO activity correlated with levels of solar radiation, and PPO activity was suggested to act as a defense against photochemical oxidation and co-occurring high populations of insect herbivores. The high temperature optimum for birch PPO may reflect a possible role of enzymes in the defense of photochemical oxidation, as in direct sunlight, the temperature of a leaf may rise surprisingly high.

The unusually fast response of birch PPOs suggests transcription- and translation-independent induction. PPOs have been reported to exist as a latent form in many plant species, and they seem to be stable proteins (Söderhäll, 1995; Thipyapong et al., 1995). We do not yet know whether birch has a latent PPO reserve, which could be induced by herbivory and high temperature. However, we used PMSF to inhibit protease activity, and the increase in activity was probably not a consequence of the release of latent PPOs. Moreover, we measured PPO activity in the absence of exogenous catalase, which can be used to remove endogenous H_2O_2. The increased PPO activity in leaves of larval twigs at +25°C may thus be a consequence of the increased endogenous level of H_2O_2, which PODs need as a co-substrate and which is known to accumulate as a response to wounding (Bi and Felton, 1995; Orozco-Cárdenas et al.,2001; Gould et al., 2002; Musetti et al., 2005; Ruuhola and Yang, 2006).

Our results may contest the use of one even temperature in bioassays. More reliable results would be obtained by using varying temperatures following the natural temperature variation. First, temperature variation between nights and days may be considerable in subarctic climate. Second, rearing temperature affects not only the growth of animals, but also the activities of defensive enzymes and may change the effects of allelochemicals on the performance of herbivores.

Our results, also, may offer a possible mechanism for the hypothesis that low spring temperature promotes the success of *E. autumnata* by providing better food via decreased defensive enzyme activities, as PPO activity was highly temperature-dependent. Moreover, both larval growth rate and PPO activity were affected after the 12-h wounding treatment, suggesting a transcription- and translation-independent induced mechanism in both cases. At +12°C, PPO activity was decreased by the wounding treatment as was the growth of larvae that contradicts that PPOs act as defensive enzymes against *E. autumnata* larvae at this temperature. However, POD activity increased slightly (albeit nonsignificantly) at this temperature and may explain the retarded growth of larvae. At +25°C, PPO activity was increased by wounding and larval growth was retarded. In another study, we found that growth, consumption, and food conversion efficiencies of the last and the most voracious fifth instar were negatively related to foliar PPO and PPO/POD activities in damaged trees, but not in control trees (Ruuhola et al. unpublished results).

Our results suggest biological mechanisms behind the interactive relations between biotic and abiotic factors in determining the resistance and suitability of plants for herbivores. However, further studies need to be conducted with different instars (and species) and different temperatures before we can get closer to an answer for the key question: can cool spring temperatures affect the population dynamics of Lepidopteran species outbreaks via improved food quality due to decreased activities of defensive enzymes? We should neither forget the effects of temperature on the third level of tritrophic

interaction, namely, diseases, parasites (see also Kapari et al., 2006), and predators; these may be more strongly affected by low temperatures than their hosts (Nuorteva and Jussila, 1967; Virtanen and Neuvonen, 1999).

Acknowledgements We thank the staff of the Kevo Subarctic Research Institute and the Department of Animal Physiology for providing facilities. We also thank Seppo Neuvonen, Irma Saloniemi and Erkki Haukioja, who offered constructive comments on the manuscript. In addition, we thank our hardworking field assistants, Netta Tolvanen, Tanja Kyykkä, Fia Haavisto, Pauliina Wäli, and Jenny Koivisto. Ellen Valle kindly checked the English language. The study was supported financially by the Academy of Finland (Project nos. 29885301, 207294, 206144), the Kone and Emil Aaltonen Foundations.

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J Chem Ecol (2007) 33:477–491
DOI 10.1007/s10886-006-9244-3

Assessment and Implications of Intraspecific and Phenological Variability in Monoterpenes of Scots Pine (*Pinus sylvestris*) Foliage

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Received: 25 August 2006 / Revised: 3 November 2006 / Accepted: 6 December 2006 /
Published online: 1 February 2007
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Abstract Scots pine populations contain individuals with widely differing amounts and composition of monoterpenes and exist as one of two chemotypes: with or without Δ^3-carene. We investigated the significance for ecological studies of two types of variation in monoterpenes: (1) the inherent variability in the concentration of monoterpenes or their relative amounts in needles of seedlings, saplings, and mature trees; and (2) phenological variation in developing needles. The relative composition of needle monoterpenes in 5-year-old saplings changed during the needle development period until the final composition was reached upon needle maturity. Changes in composition depended on chemotype. Needles of the "no-Δ^3-carene" chemotype had higher absolute concentrations of α-pinene, β-pinene, camphene, and total monoterpenes than "Δ^3-carene" chemotype. For the "Δ^3-carene" chemotype, the relative concentration of Δ^3-carene during the needle growing season and immediately after emergence of seedlings was higher compared to that reached at needle maturity. Repeated removal of single needles (at weekly intervals during growth) from 5-year-old saplings did not influence the composition of monoterpenes. Within a natural Scots pine dominated woodland, 18% of mature Scots pines (N=574) belonged to

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the "no-Δ^3-carene" chemotype. Chemotypic variation within populations means that the statistical power with which differences in monoterpene concentrations can be detected is lower when sampling from the whole population compared to sampling within chemotypes. Reduction of this background variation and accounting for chiral variation if present, would significantly aid efficiency, interpretation, and understanding of processes in chemical and ecological research. One method for achieving this is the screening of plants for chemotypes before the establishment of experiments or field sampling regimes. We present a summary of suitable analytical methods for needle tissue that facilitates this prior screening.

Keywords Absolute concentration · α-pinene · Camphene · β-pinene · Δ^3-carene · Chemotypes · Relative composition · Seasonal variation

Introduction

Monoterpenes have multiple ecological functions (Langenheim, 1994; Huber et al., 2004). For example, they can act as allelopathic agents inhibiting seed germination (Vokou et al., 2003), affect soil microbial processes including nitrification (Paavolainen et al., 1998) and nutrient cycling (White, 1994), and act as chemical defenses against insect and mammalian herbivory (Duncan et al., 1994; Linhart and Thompson, 1999). Scots pine (*Pinus sylvestris* L.) contains a large pool of monoterpenes in the resin that comprise about 1% of the dry weight of needles (Heyworth et al., 1998; Kainulainen et al., 2000a). As monoterpenes are volatile, they are continually released into the airspace surrounding the plant (Janson, 1992). Within this chemical landscape, specific enantiomers can elicit specific responses such as neuronal responses in the antenna receptor cell of several insect species including a specialist pine weevil (Wibe et al., 1998) and bark beetles (Pureswaran et al., 2004a) where they are used for host recognition (Wallin and Raffa, 2001). The defensive function of monoterpenes is thought to derive predominantly from their lipophilicity and ability to disrupt cell membrane processes (Wink, 2003), as they have limited water solubility (Weidenhamer et al., 1993). Because of this general and specific "bioactivity" and their heritability (Hiltunen, 1976), monoterpenes mediate ecological and evolutionary interactions across multiple scales (Theis and Lerdau, 2003, Iason et al., 2005).

Monoterpene composition in conifers is diverse, varying widely between (Hatcher, 1994) and within (Forrest, 1980; Latta et al., 2003; Pureswaran, et al., 2004b) species. Within populations of Scots pines, there are large concentration variations among individual trees (Forrest, 1980); analysis of isozymes showed 93% of the variation to be within and only 7% between populations (Kinloch et al., 1986). Analysis of chloroplast microsatellites attributes 89% of the genetic variation within and 11% between populations (Naydenov et al., 2005). Monoterpenes in Scots pine consist of four major and at least eight minor compounds (Sjödin et al., 2000) that vary in composition between different tissues in individual trees (Sjödin et al., 1996). There are two different chemotypes for Scots pine that refer to Δ^3-carene being either present or absent (Chalchat et al., 1985; Weissmann and Lange 1990). Constitutive variation in monoterpenes within and between Scots pine populations is genetically determined to some degree (Bohlmann et al., 1998; Phillips et al., 2003; Theis and Lerdau, 2003; Martin et al., 2004, Huber et al., 2004). However, monoterpenes in *Pinus* can also be induced by environmental factors such as herbivory (Litvak and Monson, 1998), adding to the source of variability to natural populations.

Quantification of environmental influences on the composition and concentration of monoterpenes against this background variability is fraught with methodological and statistical problems. We suggest that prior screening of experimental material or wild populations for monoterpenes can reduce background variability and increase the probability of detection of responses of concentrations of specific compounds to factors of interest. In contrast, most studies that have used monoterpenes to infer genetic variation (Hiltunen, 1976) use the relative proportions of monoterpenes (Forrest, 1980) and consider these to be constant for individual trees.

In this work, we investigated monoterpene variability in Scots pine. Specifically, we asked: (1) whether proportions of needle monoterpenes in 5-year-old saplings vary phenologically during the period of needle development; (2) how monoterpene concentrations vary with age of seedlings during their early growth; and (3) how production of individual monoterpenes is affected by chemotype in Scots pine. Based on the results, we illustrate chemical difference between the two chemotypes and advocate taking these into account for experimental designs that investigate either the effects of monoterpenes on ecological processes or environmental influences on monoterpene production. To enable preexperimental screening, we describe a technique that uses small amounts of plant material, one Scots pine needle, and we show that the sampling does not impact future monoterpene composition.

Methods and Materials

Sampling and Extraction of Monoterpenes from Needles Monoterpenes are stored in Scots pine needles within resin canals. Whole needle extraction yields only a quarter of the absolute concentration compared to cut needles; hence, needles need to be cut into sections approximately 5 mm in length to assure access to all resin canals (Lin et al., 2001). Exhaustive extraction with hexane is reached after 90 min for cut needles and 15 d for whole needles. Kieselgel 60 (Merck, Darmstadt, Germany) was used to adsorb co-extracted polar compounds and water from hexane extracts. Samples thus prepared may be stored for up to 3 mo at −20°C.

For determination of absolute concentrations, isobutylbenzene was used as internal standard. The dry weight of needles was determined after evaporation of the liquid phase at room temperature followed by drying the sample for 48 hr at 50°C.

Gas Chromatography—Determination of Low Concentrations of Monoterpenes by Using GC-MS-SIM A gas chromatography-mass spectroscopy (GC-MS) consisting of a Trace GC 2000 (ver. 1.3) and Trace DSQ mass spectrometer (ver. 1.3.0 SP4) was used. A Sol Gel 10-25 column (30 m×0.25 mm id from SGE, UK) was used for separations. Xcalibur™ (ver. 1.3). software was used for data capture and analysis. The temperature program started at 50°C (held for 1 min), followed by a temperature gradient of 3°C min^{-1} up to 75°C, followed by a gradient of 30°C min^{-1} up to 200°C (held for 2 min) to clean the column. The injection volume was 2 μl, and injections were conducted in the splitless mode. The mass spectrometer was used in single ion monitoring (SIM) mode for the 93 mass fragment only. Data were captured between 2.5 and 10 min.

The monoterpenes of Scots pine extracted and quantified with retention times and response factors for the 93 mass fragment are given in Table 1. The lowest amount of α-pinene detected with this method was 20 pg, which corresponded to 0.001 μg $(g\ DM)^{-1}$.

Table 1 Retention times and response factors for the 93 mass fragment for monoterpenes of Scots pine

Compounds	Retention Time (min)	Response Factor
α-Pinene	3.35	17.3
Camphene	3.65	9.4
β-Pinene	4.07	13.9
Myrcene	4.49	5.4
Isobutylbenzene[a,b]	4.60	1
Δ^3-Carene	4.89	16.3
α-Terpinene	5.02	7.2
Limonene[c]	5.34	7.7
γ-Terpinene	6.15	10.3
Terpinolene	7.01	6.7

[a] Internal standard

[b] α-Phellandrene co-eluting

[c] Bornyl acetate co-eluting

Gas Chromatography—Determination of High Concentrations of Monoterpenes and Their Enantiomers by Using GC-FID A Carlo Erba MFC 500 GC with flame ionization detector (FID) (T=250°C) equipped with a WCOT fused silica 25 m×0.25 mm i.d. column coated with CP WAX 52 CB (Varian, UK) was used. Injection volumes were 1 μl, and the splitting ratio was 1 to 50. Initial temperature was 70°C followed by a temperature gradient of 3°C min^{-1} up to 160°C. The following monoterpenes in order of retention were separated and identified: tricyclene, α-pinene, camphene, β-pinene, sabinene, Δ^3-carene, myrcene and α-phellandrene (co-eluting), α-terpinene, limonene, β-phellandrene, γ-terpinene, and terpinolene. A ChromQuest (ver. 2.53) chromatography workstation was used for data capture and processing. The response factor for the FID was taken as one for all monoterpenes (Raffa and Steffeck, 1988). The lowest concentration of α-pinene detected with this method was 2 ng, which corresponded to 100 μg (g DM)$^{-1}$.

For the determination of enantiomers, column coupling was used. Individual monoterpene peaks were transferred onto a CP chiralsol-Dex CB column (25 m by 0.25 mm) using a switching valve. Identification of enantiomers was based on commercial standards.

Phenological Variation in Needle Monoterpenes Ten 5-year-old Scots pine trees were sampled every week to fortnight from February to December 2004 by removing one needle of the last growing seasons cohort (referred to as "old needles"), and after late May when new needles emerged, one needle from the current seasons growth (referred to as "young needles"). Scots pines grow by forming new branches each year from buds set during the previous growing season. In this context, a branch describes the section of wood formed during the last growing season at the onset of sampling (9 February 2004). Needles were taken from different branches, and once a needle was removed, the branch was marked with polytetrafluoroethylene (PTFE) tape to prevent resampling from that branch (to avoid possible changes due to induction). During consecutive sampling occasions, branches were chosen on alternate whorls. One needle was used for extraction on each sampling occasion. The extracts were analyzed by using the GC-FID method.

Variation in Absolute Monoterpene Concentrations During Early Growth in Seedlings (3 and 8 wk Old) The absolute concentration of individual monoterpenes was determined in 3- (N=351) and 8-wk-old seedlings (N=1,076), which were grown from seed collected from the same population. Seeds were germinated in damp paper towels and transplanted into potting soil in cellular seedlings trays (3×3×5 cm). The growing medium consisted of Levingtons 2Ca compost (intermediate nutrient level, coarse, acidic) with 10% grit and a slow release fertilizer (Ficote 180) added at a rate of 4.5 kg m^{-3} (Levingtons, Ipswich,

Suffolk, UK). From each seedling, one needle was removed and used for extraction. Absolute concentrations were determined by using the GC-MS-SIM method.

Consequences of Chemotypic Differentiation: Monoterpene Concentrations in Needles of Scots Pine Saplings (3 to 12 Yr of Age) Two populations of saplings were sampled at two different locations. Population 1 was situated at latitude 57°0′ N and longitude 3°30′ E. Population 2 was located at latitude 57°3′ N and longitude 3°16′ E. Samples were collected in March and April 2003. From each sapling, ten needles were removed and extracted on-site. Samples were taken in duplicate. Monoterpenes were quantified with the GC-FID method.

Consequences of Chemotypic Differentiation: Monoterpene Concentrations in Needles of Mature Scots Pines The study site consisted of a natural Scots pine dominated woodland covering about 4 km^2 that is located at latitude 57°3′ N and longitude 3°16′ E. Needle samples were obtained between January and March 2002 and January and April 2003, by cutting off at least ten branch ends (including all the needles from the latest growth period) from at least two different main branches located on the southern side, low crown level of each of 574 mature trees. Samples were placed into ziplock plastic bags and most of the air removed before sealing. Needles were frozen on-site by placing the plastic bag between two aluminum plates kept in an ice box containing dry ice and stored at −20° C. From each Scots pine, at least 20 needles were cut and extracted (in duplicate). Monoterpenes were determined with the GC-FID method.

Statistical Analyses Mean monoterpene concentrations in the needles of the "no-Δ^3-carene" and "Δ^3-carene" chemotypes were compared in the sample of 574 mature trees, and separately, in the samples of saplings collected from "population 1" and "population 2". Distributions of these data and their residuals following analysis of variance (ANOVA) were skewed. Therefore, the descriptive statistics presented are medians and upper and lower quartiles. The *F*-statistics presented were from ANOVAs that used normalizing cube-root transformed data.

The statistical power associated with sampling needles from the whole population of trees or from only the "Δ^3-carene" or "no-Δ^3-carene" chemotypes was investigated for the population of 574 mature trees. The probability of detecting an increase in mean of 20% in α-pinene concentration, at $P<0.05$ with a *t*-test, was calculated from a simulation that involved drawing two groups with sample sizes increasing in number from 2 up to 80 per group, at random either from within the "Δ^3-carene" or "no-Δ^3-carene" chemotypes, or from the population as a whole. The simulations were run 1,000 times for each sample size. These tests were performed on cube-root transformed data, and they compared the power of detecting a difference between means on the cube-root scale that was equivalent to a 20% difference on the original untransformed scale. The rationale for comparing the effect of Δ^3-carene chemotype on needle α-pinene concentration was that intercorrelations among the monoterpenes mean that almost all monoterpenes covary with Δ^3-carene chemotypes, and α-pinene is the most abundant monoterpene.

Results

Phenological Variation in Needle Monoterpenes within Season Figure 1 shows the relative proportion of α-pinene and Δ^3-carene sampled from Scots pine trees of the "Δ^3-carene"

chemotype only, over an extended period of time. For the "old" needles, the relative concentration of α-pinene and Δ^3-carene did not change significantly during the sampling period. The relative concentrations in newly emerged needles differed from "old" needles with α-pinene being initially produced at lower relative concentration and Δ^3-carene being produced initially at higher relative concentration compared to "old" needles. For "young" needles, the relative concentrations changed until those of "old" needles were obtained late during the growing season.

By chance, eight of the ten experimental trees belonged to the "Δ^3-carene" chemotype (Figs. 1 and 2). The "no-Δ^3-carene" chemotype produced α-pinene initially in higher proportions in "young" compared to "old" needles. The relative proportion of camphene and β-pinene, the next most abundant monoterpenes, also gradually increased in newly emerging needles reaching a stable relative concentration.

Note that although the combined dataset containing both chemotypes contained a larger number of samples (Fig. 2), the relative concentrations of monoterpenes had higher standard errors of estimate, showed a less clear temporal trend in composition, and had a lower mean relative concentration of Δ^3-carene. This pattern of errors of estimate being greater in the whole population despite its larger sample size, compared with those in the restricted sample of the Δ^3-carene chemotype, is also seen by comparing the interquartile ranges of α-pinene and Δ^3-carene concentrations in Tables 3 and 4. The consequences of the greater variance (in this case α-pinene) in the overall population compared to the two constituent chemotypes are illustrated in the analysis of the statistical power associated with sampling different group sizes from the monoterpene analyses of needles from 574 mature trees (Fig. 3). The analysis shows that the probability of detecting a difference in 20% between the means of two samples is lower for any given group size, when the comparison

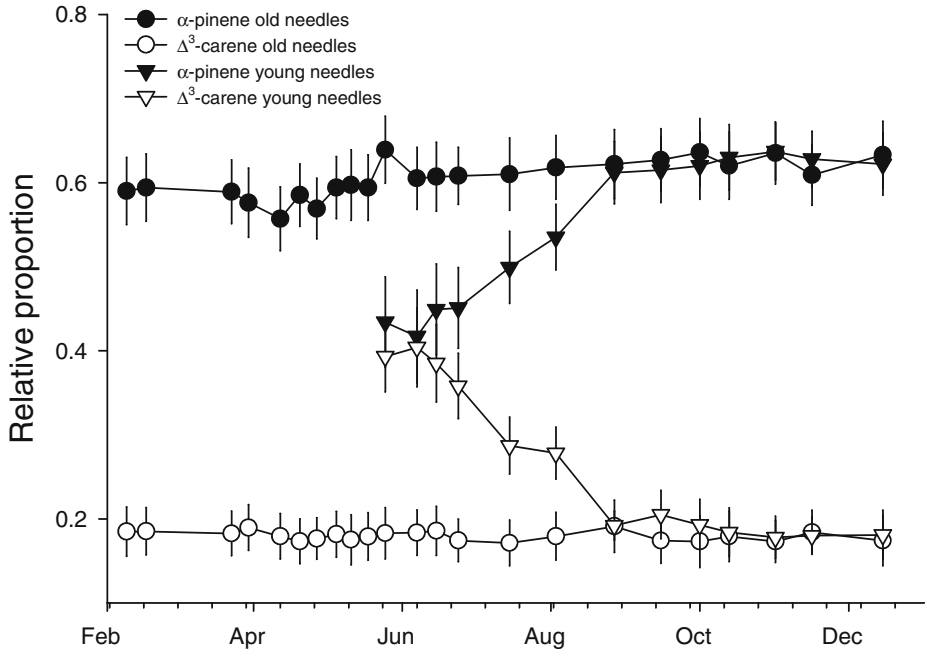


Fig. 1 Relative proportion of α-pinene and Δ^3-carene in old and young needles of Scots pine for the chemotype containing Δ^3-carene. *Bars* are one standard error (N=8)

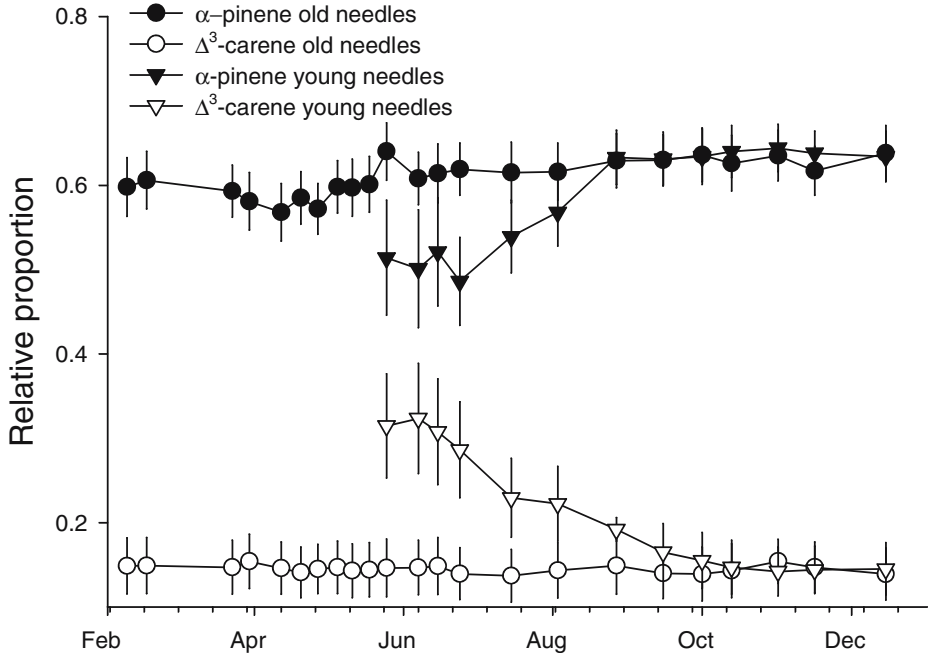


Fig. 2 Relative proportion of α-pinene and Δ^3-carene in old and young needles of Scots pine. *Bars* are one standard error (N=10)

is being made within the overall population, compared to the groups being drawn from within either of the two chemotypes (Fig. 3). The sample size required to maximize statistical power is larger in the case of the whole population.

Screening of Seedlings The maximum and mean concentrations of monoterpenes measured for 3- and 8-wk-old seedlings, respectively, is given in Table 2. The mean and maximum absolute concentrations of individual and total monoterpenes determined in the seedlings increased by a factor of 4 between wk 3 and 8. For the two major monoterpenes, the mean relative concentration for α-pinene was with about 30%, considerably lower than 54% obtained for mature Scots pine. For Δ^3-carene, the relative concentration for seedlings was about 45%, which was considerably higher than the mean of 25% obtained for mature Scots pines.

Characteristics of Chemotypes in Saplings and Mature Scots Pines For the 574 mature Scots pines, α-pinene and Δ^3-carene contributed 75.9% of the total needle monoterpenes with 49.9% for α-pinene and 26.0% for Δ^3-carene (median values). β-Pinene and camphene were the third and fourth most abundant monoterpenes with relative concentrations of 5.2 and 4.3%, respectively. However, as the whole population consists of two chemical subpopulations, these relative concentrations depend on the chemotype (Table 3). The "no-Δ^3-carene" chemotype contains 76.8% α-pinene, 0% Δ^3-carene, 10.1% β-pinene, and 4.7± 0.2% camphene as relative concentrations. This contrasts with the "'Δ^3-carene" chemotype, which contained 45.0% α-pinene, 29.8% Δ^3-carene, 4.5% β-pinene, and 4.1% camphene. The "no-Δ^3-carene" chemotype comprised 11.5 and 14.4% of the saplings sampled in

Table 2 Maximum and mean ± standard error for the monoterpene concentrations [μg (g DM)$^{-1}$] determined for Scots pine seedlings sampled at two different ages

Compounds	3 Wk Old		8 Wk Old	
	(*N*=351)		(*N*=1,076)	
	Maximum	Mean ± SE	Maximum	Mean ± Se
α-Pinene	20.05	0.651±0.105	83.50	2.748±0.198
Camphene	0.183	0.004±0.001	3.59	0.026±0.004
β-Pinene	4.03	0.112±0.018	18.78	0.429±0.029
Myrcene	7.39	0.137±0.029	22.64	0.557±0.038
Δ^3-Carene	37.62	0.748±0.150	126.65	4.446±0.272
α-Terpinene			43.12	0.110±0.054
Limonene[a]	6.29	0.067±0.020	10.82	0.403±0.026
γ-Terpinene			3.40	0.072±0.006
Terpinolene	6.45	0.088±0.023	30.42	0.728±0.056
Total monoterpenes	58.06	1.806±0.291	225.04	9.519±0.553

[a] Bornyl acetate co-eluting

populations 1 and 2, respectively (Table 4), whereas for the mature Scots pine population, its proportion was 17.6% (Table 3).

There were notable differences in absolute amounts of almost all the individual and the total monoterpenes between the chemotypes, and these differences drove the observed differences in the relative proportions of the monoterpenes reported above. Separation of populations by chemotype (Tables 3 and 4) showed that the "no-Δ^3-carene" chemotype had consistently higher concentrations of α-pinene, β-pinene, and camphene in all three populations (Tables 3 and 4; ANOVA: $P<0.001$). These greater concentrations of the most abundant monoterpenes mean that the concentrations of total monoterpenes in the "no-Δ3-

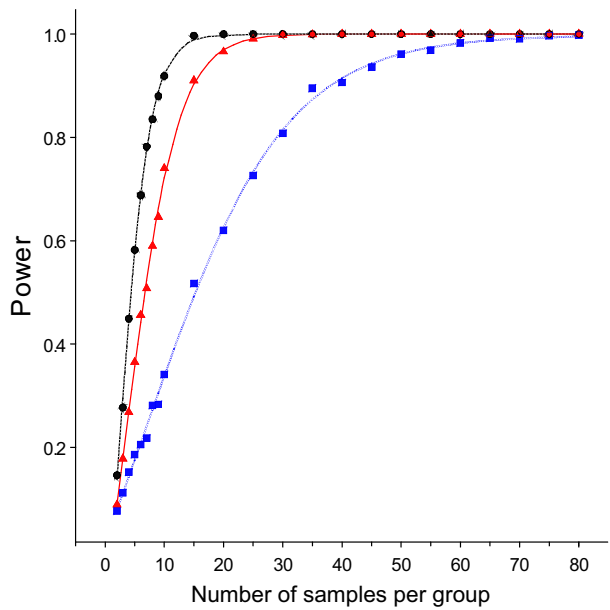


Fig. 3 The relationship between the statistical power, with which a *t*-test will detect a 20% increase in mean of α-pinene concentration, and the number of samples per group, for a comparison of two groups, drawn at random from within the "no-Δ3-carene" chemotype (-•-), the "Δ^3-carene" chemotype (-▲-), or the entire population (...■...)

Table 3 Median, upper, and lower quartiles of concentrations of needle monoterpenes for mature Scots pine trees for the whole population and separated into the two chemotypes with respect to Δ^3-carene

Populations	α-Pinene	Δ^3-Carene	β-Pinene	Camphene	Total
Whole Population (N=574)					
Q1	2.243	1.184	0.173	0.220	5.746
Median	3.584	1.869	0.373	0.306	7.194
Q3	5.538	2.614	0.751	0.448	8.986
"No-Δ^3-Carene" Chemotype (N=101)					
Q1	3.998	0.004	0.322	0.284	5.751
Median	5.656	0.005	0.741	0.391	7.364
Q3	8.180	0.008	1.155	0.542	10.062
"Δ^3-Carene" Chemotype (N=473)					
Q1	2.064	1.593	0.159	0.206	5.790
Median	3.234	2.145	0.326	0.292	7.193
Q3	4.896	2.868	0.677	0.423	8.884

Concentrations are in mg (g DM)$^{-1}$.

carene" chemotype were actually higher than those of the "Δ^3-carene" chemotype (Tables 3 and 4). Although the greater concentration in total monoterpenes in the "no-Δ3-carene" chemotype was not significant in mature trees (ANOVA: *NS*, Table 3), the effect was statistically significant in saplings from population 1 (ANOVA $P<0.001$, Table 4) and population 2 (ANOVA: $P<0.05$, Table 4).

In the needles from the 574 mature trees, there were significant ($P<0.001$) and near linear correlations between tricylene and camphene ($r=0.980$) and Δ^3-carene and terpinolene ($r=0.978$). α-Pinene was correlated with limonene ($r=0.725$, $P<0.001$) and was the best predictor of total monoterpenes ($r=0.820$, $P<0.001$). α-, γ-terpinene, and sabinene were correlated with Δ^3-carene ($r=0.756$, $r=0.793$, $r=0.722$), respectively, with $P<0.001$ for all. These correlations were confirmed by using the two populations of saplings even though the correlation coefficients differed slightly.

Enantiomeric Composition The enantiomeric composition of individual monoterpenes also showed variability. In the needles of a subset of the mature Scots pine population (N=143), chiral analysis showed that only four were enantiomeric. The mean percentage composition was, in three cases, dominated by a single enantiomer; α-pinene, the most abundant monoterpene, existed predominantly in the form of (+)-α-pinene [74.4±1.4% (mean± SEM), Fig. 4]. β-Pinene and camphene were dominated by (−)-β-pinene (84.7±0.8%), and (−)-camphene (84.4±0.5%), respectively. The enantiomers of limonene occurred in approximately equal proportions (53.6±0.8% (+)-limonene).

Discussion

This study uses five independent datasets to illustrate the variability of monoterpene concentration and composition depending on phenology and chemotype in populations of Scots pine. In particular, we found consistent trends of higher relative concentration of Δ^3-carene in growing needles for the "Δ^3-carene" chemotype and higher total monoterpene concentration in the "no-Δ^3-carene" chemotype in mature needles. The effects exerted by pine monoterpenes on associated organisms depend on the monoterpene proportionate

Table 4 Median, upper, and lower quartiles of concentrations of needle monoterpenes for Scots pine saplings (populations 1 and 2), separated into the two chemotypes with respect to Δ^3-carene; concentrations[a] are in mg (g DM)$^{-1}$

Populations	α-Pinene	Δ^3-Carene	β-Pinene	Camphene	Total
Whole Population 1 (*N*=278)					
Q1	0.269	0.108	0.048	0.068	0.737
Median	0.548	0.235	0.079	0.108	1.185
Q3	0.957	0.386	0.156	0.155	1.873
"No-Δ^3-carene" chemotype (*N*=40)					
Q1	0.653	0.001	0.073	0.107	1.054
Median	0.925	0.001	0.116	0.144	1.375
Q3	1.470	0.002	0.210	0.197	2.106
"Δ^3-carene" chemotype (*N*=238)					
Q1	0.256	0.169	0.045	0.065	0.713
Median	0.475	0.269	0.072	0.100	1.156
Q3	0.829	0.440	0.145	0.147	1.842
Whole Population 2 (*N*=277)					
Q1	0.315	0.138	0.051	0.072	0.794
Median	0.559	0.235	0.089	0.108	1.252
Q3	0.940	0.394	0.163	0.155	1.774
"No-Δ^3-carene" chemotype (*N*=32)					
Q1	0.748	0.001	0.073	0.117	1.124
Median	1.016	0.001	0.106	0.147	1.484
Q3	1.712	0.002	0.210	0.196	2.303
"Δ^3-carene" chemotype (*N*=245)					
Q1	0.285	0.174	0.050	0.069	0.763
Median	0.502	0.257	0.086	0.103	1.178
Q3	0.840	0.415	0.156	0.150	1.750

[a] As whole needles were extracted, absolute concentrations are only about a quarter of those using cut needles.

composition (Iason et al., 2005; Pakeman et al., 2006; Thoss and Byers, 2006), which we show to be dependent on the chemotype.

The inheritance of monoterpene synthesis (Hiltunen, 1976), with the "no-Δ^3-carene" chemotype being considered the double recessive genotype (Kinloch et al., 1986), is expressed through monoterpene synthases (Huber et al., 2004; Martin et al., 2004). The significant correlations between individual monoterpenes within different Scots pine populations supports the existence of fewer monoterpene synthases than individual monoterpenes produced (Davis and Croteau, 2000; Schwab, 2003). Camphene co-occurred with tricyclene and Δ^3-carene co-occurred with terpinolene, α- and γ-terpinene, and sabinene. These correlations mostly agree with the monoterpene biosynthesis in Scots pine as described by Juvonen (1966, quoted in Hiltunen, 1976), apart from sabinene that seems to co-occur with Δ^3-carene rather than with β-phellandrene. Two different biosynthetic pathways have been described for β-pinene and might underlie the reason for the weaker correlations between β-pinene and all other monoterpenes. The enantiomeric composition of needles from mature Scots pines showed a disproportionately high production of (+)-α-pinene, (−)-β-pinene, and (−)-camphene, while limonene was racemic (Sjödin et al., 1996, 2000). On the basis of the biosynthetic pathways, determination of the concentrations of α- and β-pinene, camphene, and Δ^3-carene should suffice for preexperimental screening for Scots pine because: (1) they had significant correlations

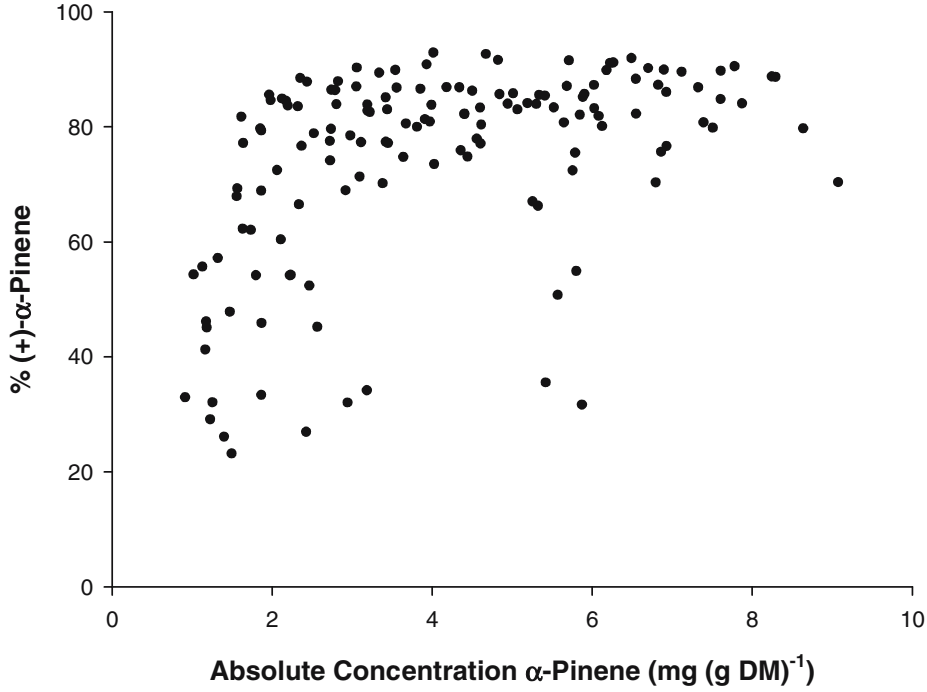


Fig. 4 The percentage of the (+)-α-pinene enantiomer vs the absolute total concentration of α-pinene, in needles of 143 mature Scots pine trees

with those monoterpenes that occurred in minor relative proportions, and (2) their sum comprised 79 to 94% of total monoterpenes.

For Scots pine, one mature needle can be used for preexperimental screening, as its content represents predominantly constitutive monoterpene production. No variation in the composition of monoterpenes occurred between different year-classes of mature needles or time of sampling. Even if the most relevant tissue for a future experimental investigation differs from needles, we advocate the usage of needles for preexperimental screening, as it allows determination of the chemotype that applies to other aboveground tissues of Scots pine (Thoss, unpublished data).

The "no-Δ3-carene" chemotype did not have lower concentrations of total monoterpenes than the "Δ^3-carene" chemotype. In fact, in the plants where Δ^3-carene was absent, the concentration of total monoterpenes was higher, suggesting that the absence of Δ^3-carene was compensated for by other monoterpene synthases. With regard to the chemical characteristics of the two chemotypes, it was surprising to note in the "Δ^3-carene" chemotype that the particular compound had been produced in higher relative concentrations in seedlings and during needle development compared to the composition at needle maturity. We speculate that there may be evolutionary reasons for this allocation of resources to a relatively high proportion of Δ^3-carene in very young developing pine needles; it may be associated with a higher biological activity and protective function (Ikeda et al., 1977). α-Pinene and Δ^3-carene differ in their chemical reactivity with autoxidation and polymerization occurring at lower temperature and at higher yields for Δ^3-carene compared to α-pinene (Rothenberg et al., 1998). These differences in reactivity

could explain the lack of occurrence of chemotypes consisting predominantly of Δ^3-carene, as the oleoresin could polymerize within the resin canal system and negatively impact the defensive function of oleoresin that relies on its fluidity.

Relative proportions of monoterpenes in plants have been used to define species characteristics (von Rudloff, 1975; Sjödin et al., 1996, 2000; Latta et al., 2000, 2003) and population genetics (Forrest, 1980; Naydenov et al., 2005). They also have been utilized in ecological studies (Delorme and Lieutier, 1990; Sadof and Grant, 1997; Tiberi et al., 1999; Barre et al., 2003; Nerg et al., 2004). Absolute concentrations are the preferred measure where either some form of dose-dependency is thought to underlie the processes being studied (Duncan et al., 1994; Iason et al., 1996; Litvak and Monson, 1998) or where an ecophysiological response of pines to an environmental manipulation is investigated (Kainulainen et al., 1996, 2000a,b; Heyworth et al., 1998; Lamontagne et al., 2000, 2002; Sallas et al., 2003). Quantification of monoterpene enantiomers appears to be important in the study of specific interactions with specialist insects (Wibe et al., 1998; Pureswaran et al., 2004a). Nonetheless, the role of monoterpenes in Scots pine resistance to specialist insects (Sadof and Grant, 1997, Barre et al., 2003, Nerg et al., 2004) and in other broader ecological functions (see Introduction) has also been identified without recourse to analysis of enantiomers. We know of few, if any, studies in which the intra- and interpopulation variation of monoterpenes, satisfactorily takes into account chirality of compounds based on large sample sizes, and we advocate that this deficiency should be corrected. It is clear that either or both the measurement of relative proportions and absolute concentrations of monoterpenes, with and without chiral separations, are of utility, but the most suitable analysis, of course, depends on the aims of the study in question.

A random selection of Scots pine trees is likely to result in a mixture of two chemotypes: the "no-Δ^3-carene" chemotype, which occurs in about every fifth tree of mature Scots pines, and the "Δ^3-carene" chemotype. It is possible that the reason that a few studies that investigate the causal relationship of environmental manipulations on absolute monoterpene production (Kainulainen et al., 1996, 2000a,b; Heyworth et al., 1998; Lamontagne et al., 2000, 2002; Sallas et al., 2003) have found any significant responses for monoterpenes is because the existence of the two chemotypes was not accounted for in the experimental designs. The effects of chemotypic variation on the concentrations of Δ^3-carene itself are likely to be profound, as its values are zero for about 20% of the population. In addition, this study demonstrates the consequences for statistical sampling of the variation in the concentrations or proportions of other monoterpenes, which also vary between the Δ^3-carene chemotypes. Although our statistical treatment did not specifically include chiral differences, the predominance of particular enantiomers means that our conclusions regarding the statistical consequences of variability, e.g., for selection of samples, are also likely to hold when the different enantiomers are taken into account.

Based on these observations, we suggest that the effects of different chemotypes should be incorporated into experimental designs and sampling programs for chemical ecology studies, and we describe a suite of rapid sampling and analysis techniques that will enable this preexperimental screening. The effects of contrasting enantiomers should also be considered in studies in which compounds exist as enantiomers in varying proportions and in which their specific effects may be important. Furthermore, unless contrasting treatments can be applied within individuals or by controlling for genetically based variation by other means, such as cloning, then a statistically rigorous sampling regime, with adequate replication, should be applied to studies of chemically variable systems such as monoterpenes.

Acknowledgements We thank the Scottish Executive Environment and Rural Affairs Department for funding this study; Joan Beaton and Diane Smith for raising the seedlings and other assistance; David Elston of Biomathematics and Statistics Scotland for advice; and Bob Mayes, Simon Thirgood, and anonymous referees for critically reviewing the manuscript. We thank the Balmoral Estate and the National Trust for Scotland, Mar Lodge Estate for access to the study sites. JOR would like to acknowledge support by the Australian Research Council and the Joseph William Gottstein Memorial Trust fellowship, and traveling grants by the Journal of Experimental Biology and the Society of Experimental Biology.

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J Chem Ecol (2007) 33:493–500
DOI 10.1007/s10886-006-9248-z

Effect of Host Plant Xylem Fluid on Growth, Aggregation, and Attachment of *Xylella fastidiosa*

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Received: 8 September 2006 / Accepted: 18 December 2006 /
Published online: 25 January 2007
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Abstract In the Temecula Valley of California the proximity of citrus groves to vineyards influences the incidence and severity of Pierce's disease (PD) in grapes, a disease caused by the gram-negative bacterium, *Xylella fastidiosa*. Although the glassy-winged sharpshooter (GWSS), the major insect vector of the bacterium, feeds on and moves back and forth between citrus groves and vineyards, there are no visible symptoms of disease caused by *X. fastidiosa* in citrus. Previous evidences suggested that while grapevines are susceptible to the PD strain of *X. fastidiosa*, citrus trees are resistant or tolerant but could be a reservoir to harbor the pathogen for the GWSS acquisition. We investigated the mechanisms of host plant resistance/susceptibility by examining the *in vitro* effect of xylem fluid from grapefruit, orange, lemon, and grape on the growth, aggregation, and attachment of a *X. fastidiosa* strain isolated from grape. Our results revealed that xylem fluid from grapefruit, orange, and lemon trees caused the bacterial cells to form aggregations of large whitish clumps, whereas the xylem fluid from grape vines created a visible thick biofilm. The densities of *X. fastidiosa* cells in grapefruit xylem fluid treatment were significantly higher at 6, 8, and 9 d postinoculation compared with those in grape xylem fluid treatment. The cell densities of *X. fastidiosa* cultures in orange or lemon xylem fluid were generally lower than those in grape xylem fluid treatment, whereas citrus xylem fluid significantly inhibited *X. fastidiosa* biofilm formation compared to grape xylem fluid.

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Keywords *Xylella fastidiosa* · Xylem fluid · Citrus · Grapes

Introduction

Xylella fastidiosa is a plant pathogenic bacterium that reproduces in the xylem and plugs the water-conducting tissue of grape wines, leading to Pierce's disease (PD) (Davis and Thomson, 1977; Purcell 1981). The losses because of PD in California are severe and in Temecula Valley alone growers have lost about 30% of their vineyards. In Southern California, *X. fastidiosa* is mainly vectored by the glassy-winged sharpshooter (GWSS) *Homalodisca coagulata*. The GWSS is native to the southeastern United States and northern Mexico and was first detected in California in 1989 (Sorensen and Gill, 1996). Although the insect has a broad host range, comprising many plant species in 35 families (Turner and Pollard, 1959), the major crop hosts in Temecula Valley are citrus and grapes. Previous studies in California had identified 94 plant species in more than 28 plant families as hosts of *X. fastidiosa* (Freitg 1951; Turner and Pollard, 1959; Raju et al., 1980, 1983; Sorensen and Gill, 1996; Costa et al., 2004). Most of them are asymptomatic but serve as inoculum sources of *X. fastidiosa* for vector acquisition (Purcell and Saunders, 1999).

Studies in the Temecula Valley revealed that proximity of citrus groves to vineyards has a positive effect on the incidence and severity of PD in grapes (Perring et al., 2001). The disease incidence and severity are greatest in grapevines adjacent to citrus, and decline with increasing distance from citrus (Perring et al., 2001). Several PD strains of *X. fastidiosa* were previously isolated from sweet orange trees in Florida (Hopkins et al., 1978) and by the use of insect vectors, the bacterium was transmitted from these trees to grapevines (Hopkins et al., 1978). This indicates that the PD strain of *X. fastidiosa* could survive and colonize in citrus plants and could be transmitted from citrus plants to grapevines. Although the GWSS feeds on and moves back and forth between citrus and grape plants in the Temecula Valley, there are no apparent *X. fastidiosa* disease symptoms in citrus. This suggests that citrus plants are resistant or tolerant to the PD strain of *X. fastidiosa* but may serve as a reservoir to harbor the pathogen for GWSS acquisition. Because little is known about the mechanisms involved in host plant resistance/susceptibility to *X. fastidiosa* in the system, additional information is required to determine if citrus can be a suitable reservoir for the PD strain of *X. fastidiosa*.

Biofilm is a form of microbial growth in which the cells embedded in an adhesive polysaccharide matrix aggregate and attach to a surface usually in a wet environment (Costerton et al., 1995). Biofilm is common in nature in which many important microbial processes take place. For example, biofilm formation is required for pathogenesis in many microbial species such as *Pseudomonas aeruginosa* in cystic fibrosis and oral bacteria in gingivitis (Parsek and Singh, 2003; Crossman and Dow, 2004; Scheie and Petersen, 2004). *Xylella fastidiosa* aggregates to form biofilm inside its host plants and vectors (Marques et al., 2002), and this phenomenon was implicated in *X. fastidiosa* pathogenesis (Newman et al., 2004). Recently, a defined medium was developed for *X. fastidiosa* based on the chemistry of xylem fluid of the susceptible grape cultivar Chardonnay (Leite et al., 2004). This reported medium supported better *X. fastidiosa* growth and stimulated bacterial aggregation and biofilm formation *in vitro* (Leite et al., 2004). However, the effect of xylem fluid of citrus on *X. fastidiosa* growth, aggregation, and biofilm formation remains unknown. The objective of this study was to determine the effect of host plant xylem fluid on *X. fastidiosa* growth, aggregation, and attachment to abiotic surfaces.

Methods and Materials

Experimental Plots and Collection of Xylem Fluid Commercial citrus (grapefruit, lemon, and orange) groves in proximity to vineyards were selected in the Temecula Valley, California. Three blocks of 30 citrus and 30 grape plants were used. Fifteen plants of each species were randomly selected from each block for xylem fluid extraction. Collection of xylem fluid was done between 6:00 and 10:00 A.M. in October of 2003 by using a pressure chamber apparatus as described previously (Anderson et al., 1989, 1992). Five terminal shoots from each plant were used for xylem extraction, as these are the preferred feeding sites for GWSS on plants (Mizell and French, 1987; Bi et al., 2005). Upon collection, the xylem fluid in microcentrifuge tubes was immediately placed on dry ice before final storage in a −80°C freezer until final use.

Bacterial Growth, Aggregation, and Attachment Assays A *X. fastidiosa* strain A05 (isolated from infected grape vines in the Temecula Valley) was used in this study (Costa et al., 2004). The bacterial growth was measured at an optical density of 600 nm with a spectrophotometer (Beckman Coulter DU 640). A fresh *X. fastidiosa* strain A05 culture was inoculated into PD3 medium (Davis et al., 1980), supplemented with xylem fluid (xylem fluid/medium=1:1) in borosilicate glass culture tubes on a rotary shaker at room temperature (around 24°C). At 4, 5, 6, 7, 8, 9, and 10 d after inoculation, the tubes were vortexed for 1 min to homogenize the cell suspension. Silwet-L-77 (given by OSI Specialties of GE Silicones) was added to each tube (with a final concentration of 0.001%) to disperse the bacterial cells just before homogenization. The absorbance was then measured at 600 nm. At 10 d after inoculation, the culture was photographed to record aggregation.

The effect of xylem fluid on *X. fastidiosa* attachment was evaluated by measuring biofilm formation of the bacterial cells grown *in vitro*. Formation of biofilm on the abiotic surfaces was assayed as described by Espinosa-Urgel et al. (2000). Briefly, cells of *X. fastidiosa* strain A05 were inoculated at an initial A_{600} of about 0.1 into PD3 medium supplemented with xylem fluid at 25 and 50% (v/v). The inoculum was then dispensed into polystyrene microtiter plates and incubated at 24°C. At 2, 4, and 6 d after incubation, one of the plates was taken out for processing. Each well was gently washed with deionized water, and stained with 1% (wt/vol) crystal violet. After 15 min at room temperature, the wells were washed thoroughly until there was no more residual stain in the well except that absorbed by the attached bacteria. After drying, the stain was solubilized in ethanol. The absorbance of the resulting solution was measured at 600 nm.

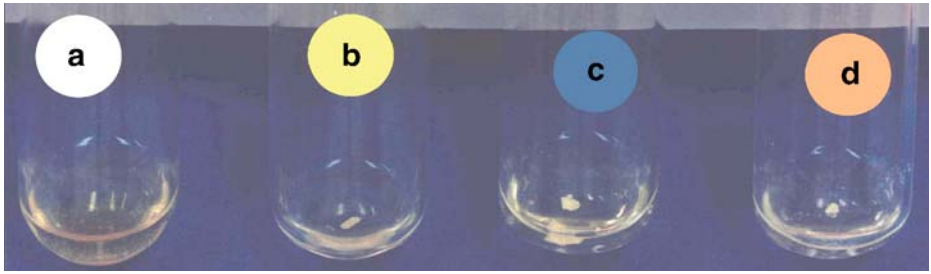


Fig. 1 Effect of host plant xylem fluid on *X. fastidiosa* aggregation. PD3 medium was supplemented with host plant xylem fluid. Tubes a, b, c, and d were each supplemented with xylem fluid from grape, grapefruit, orange, and lemon, respectively. Note the white clumps of *X. fastidiosa* aggregates formed in the grape fruit, orange, and lemon xylem fluid treatments

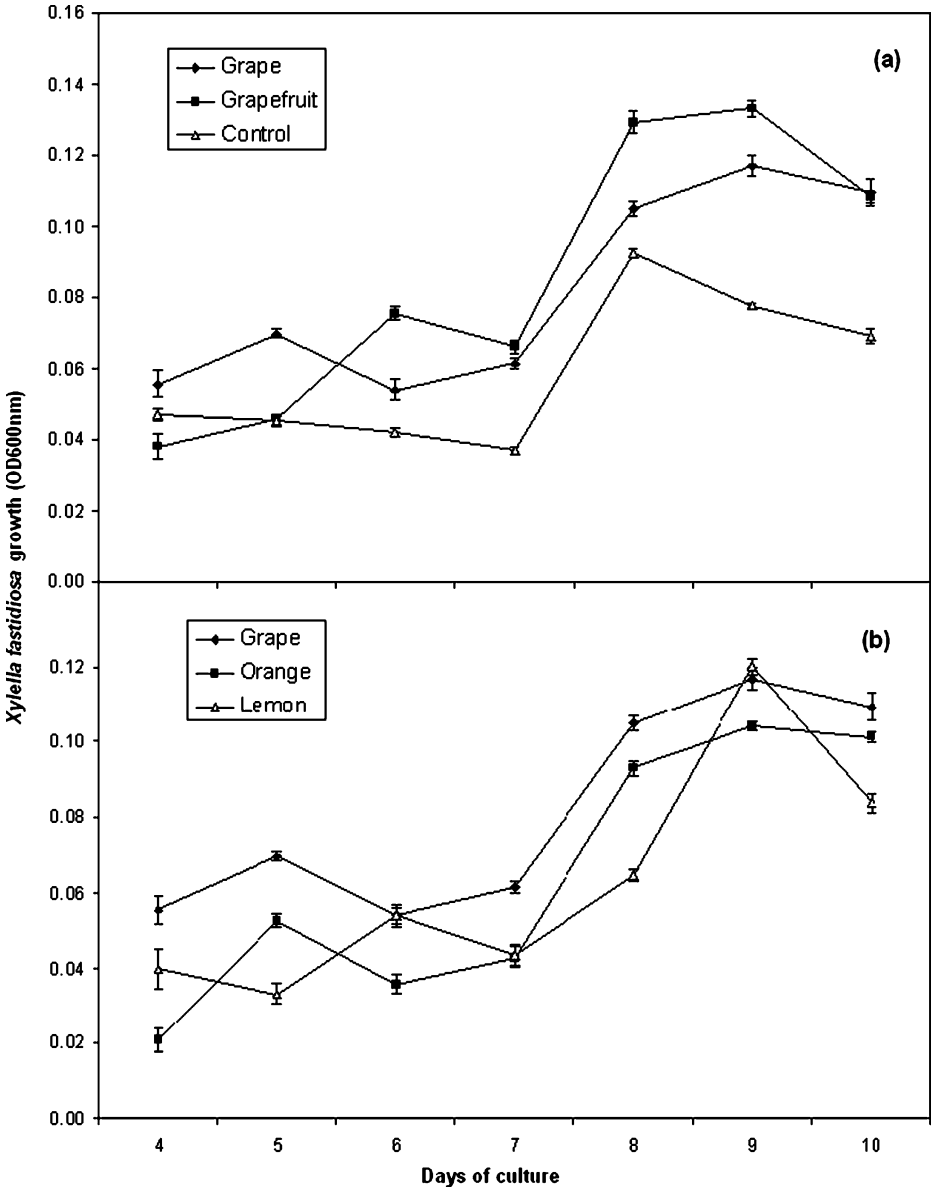


Fig. 2 Effect of host plant xylem fluid on *X. fastidiosa* growth. *X. fastidiosa* was inoculated into PD3 medium supplemented with xylem fluid from the indicated host plants. Control was *X. fastidiosa* growth in PD3 medium alone. The growth of the cultures was recorded by uniformly suspending the cells in the culture and measuring the absorbance at 600 nm

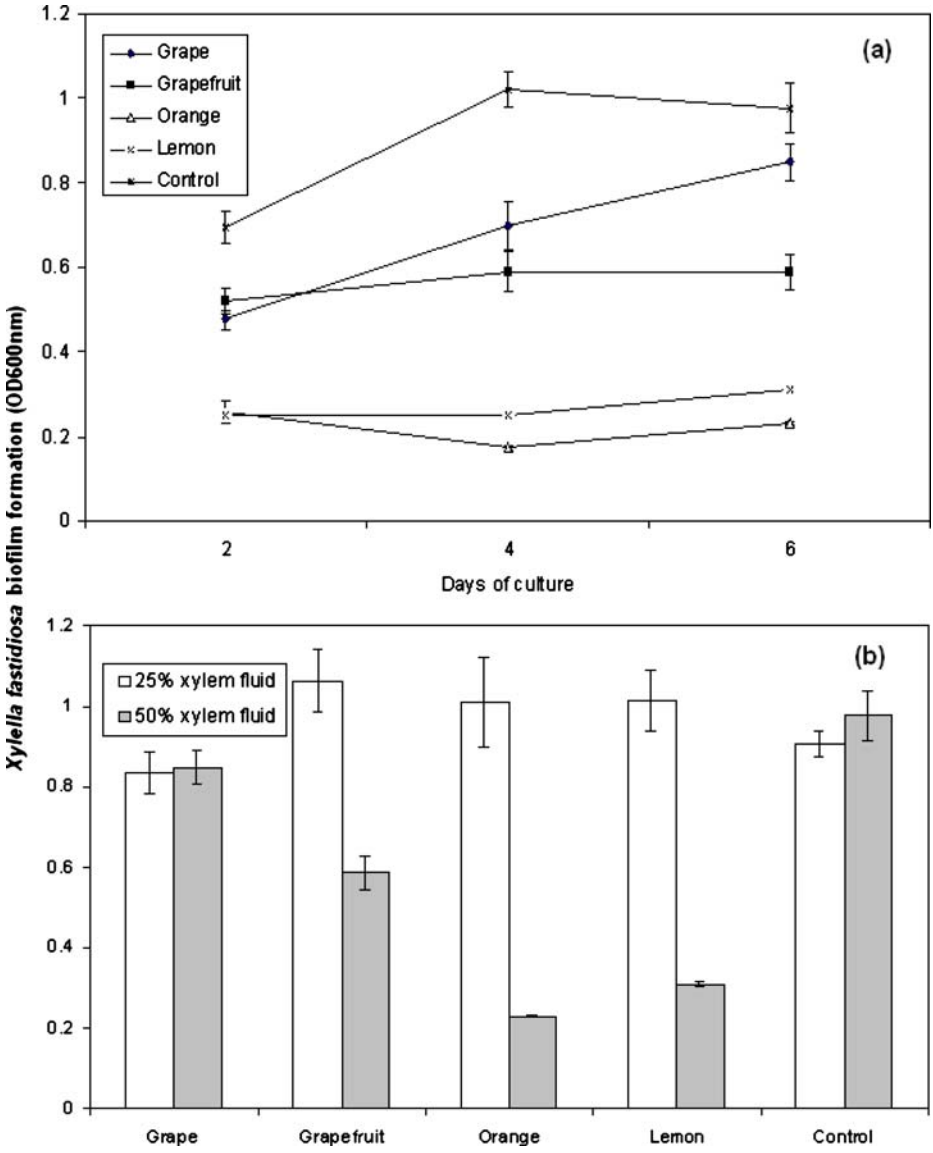


Fig. 3 Effect of host plant xylem fluid on *X. fastidiosa* biofilm formation. Biofilm formation was measured in microtiter plates as described in the "Materials and Methods" section. Biofilm cells were stained with crystal violet, the amount of stain absorbed was dissolved in ethanol, and the resulting absorbance was measured. **(a)** PD3 medium supplemented with xylem fluid at 50% (v/v). **(b)** Optic density was measured at d 6 after the culture

Statistical Analyses Analysis of variance (ANOVA) and the least significant difference test in one-way completely randomized ANOVA in SAS (SAS Institute Inc. 1999–2001) were used to analyze the resulting data and separate the means.

Results

In xylem fluid of grapefruit, orange, and lemon, *X. fastidiosa* A05 cells aggregated to form large whitish clumps suspended in the culture, leaving the culture medium clear with no observable turbidity (Fig. 1b–d); in contrast, grape xylem fluid inoculated with the same *X. fastidiosa* culture did not cause visible clumping, but rather a visibly thick biofilm was formed on the surface of glass tube and the culture was turbid (Fig. 1a). After dispersion of the clumps with Silwet-L-77, we found that the absorbance of *X. fastidiosa* culture in the grapefruit xylem fluid treatment was 14–40% higher after 6, 8, and 9 d of culture ($F_{4,35}$= 48.98, 122.41, and 105.25, respectively, $P<0.001$) compared with that in the grape xylem fluid treatment (Fig. 2a). The absorbance of *X. fastidiosa* culture in orange xylem fluid treatment was 8–168% less at all culture test periods (from d 4 to 10) ($F_{4,35}$=12.67, 55.43, 48.98, 18.49, 122.41, 105.25, and 53.21, respectively, $P<0.001$), whereas the absorbance of *X. fastidiosa* culture in lemon xylem fluid treatment was 31–110% less after 4, 5, 7, 8, and 10 d of culture ($P<0.001$) in comparison with the absorbance of *X. fastidiosa* culture in grape xylem fluid treatment (Fig. 2b).

Xylem fluid of the citrus species generally inhibited biofilm formation by *X. fastidiosa* A05 compared to that of grape (Fig. 3a). In 2-d-old cultures, lemon and orange xylem fluid treatments suppressed the amount of *X. fastidiosa* biofilm by nearly twofold ($F_{4,35}$=48.51, $P<0.001$). In 4-d-old cultures, xylem fluid of lemon and orange reduced *X. fastidiosa* biofilm formation by 2.8- and 4.0-fold ($F_{4,35}$=80.38, $P<0.001$), respectively. In 6-d-old cultures, xylem fluid of grapefruit, lemon, and orange decreased the *X. fastidiosa* biofilm formation by 1.4-, 2.7- and 3.7-fold ($F_{4,35}$=75.01, $P<0.001$), respectively. Compared with the 25% xylem fluid cultures at 6 d after inoculation, 50% xylem fluid of grapefruit, orange, and lemon decreased *X. fastidiosa* biofilm formation by 1.8- to 4.4-fold ($F_{4,35}$=28.65, 48.39, and 84.78, respectively, $P<0.001$) whereas 50% grape xylem fluid had no significant effect ($F_{4,35}$=0.05, P=0.83) (Fig. 3b).

Discussion

Xylem fluid of grapefruit, lemon, and orange can support *X. fastidiosa* growth, suggesting that these citrus species are suitable hosts for *X. fastidiosa* and may serve as a reservoir of the pathogen for GWSS acquisition in Temecula Valley. Citrus species were shown to be favorable hosts of GWSS in southern California, although they remain asymptomatic for *X. fastidiosa* infection (Bi et al., 2005; Castle et al., 2005). It is possible that GWSS transmits *X. fastidiosa* between citrus and grape plants in the course of feeding on and moving back and forth between these crops. With respect to citrus in the Temecula Valley, grapefruit constitutes the largest acreage, and its xylem fluid is most conducive to growth of *X. fastidiosa*. The severe PD incidences in grapevines close to citrus may be caused by multiplication of the pathogen in citrus and further transmission to grapevines.

Our observation of biofilm inhibition by citrus xylem fluid and stimulation by grape xylem fluid is consistent with PD strains of the bacterium not causing disease in citrus whereas remaining virulent in grapes. The results of this study appear to provide a link

between pathogenesis and biofilm formation, whereas at the same time explaining (at least partly) why the local PD strain is not pathogenic to citrus species. The formation of biofilm is considered a major virulence factor in many bacterial pathogens of plants, including *X. fastidiosa* (Marques et al., 2002).

Our results suggest that the growth, aggregation, and biofilm formation of the *X. fastidiosa* in the xylem fluid of citrus and grape were mediated by the xylem fluid chemistry. *X. fastidiosa* is a nutritionally fastidious bacterium (Wells et al., 1987). It was found that in defined medium certain amino acids such as asparagines, cysteine, glutamine, glycine, serine, and threonine are essential for *X. fastidiosa* growth; glucose stimulates the growth, whereas fructose and sucrose have an inhibiting effect (Wells et al., 1987; Chang and Donaldson, 2000). Amino acid profiles and sugar contents in xylem fluid of grape and citrus are different (Anderson et al., 1989; Bi et al., 2005). It is unknown whether differences in amino acid profiles and the sugars in the xylem fluid may differentially affect growth, aggregation, and biofilm formation of the *X. fastidiosa*. Redox status also affects the tendency for *X. fastidiosa* aggregation and biofilm formation. Adding reducing agents such as glutathione to artificial medium promotes *X. fastidiosa* aggregation and biofilm formation (Leite et al., 2004). It was reported that thiols mediate the aggregation and adhesion of *X. fastidiosa* (Leite et al., 2002). Thiol-containing compounds in xylem fluid include cysteine, methionine, and glutathione. The redox status in citrus and grape xylem fluid and its role in *X. fastidiosa* aggregation and biofilm formation, and host plant resistance/susceptibility to PD need to be further investigated.

Our findings that the xylem fluid of grape, a symptomatic host, and citrus species, asymptomatic hosts, of *X. fastidiosa*, have different effects on *in vitro* activities of the bacterium have ramifications for the control of PD. New avenues are now open for the exploitation of plant xylem fluid chemistry in the studies of *X. fastidiosa*/plant and *X. fastidiosa*/insect interactions to develop host plant resistance as component of PD management strategies. Experimentation is underway to identify the specific chemical constituents of the xylem fluid, as well as the differences in their concentrations, among the various plants species that are hosts to *X. fastidiosa*, and their roles in host plant resistance to PD.

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J Chem Ecol (2007) 33:501–512
DOI 10.1007/s10886-006-9250-5

An Isorhamnetin Rhamnoglycoside Serves as a Costimulant for Sugars and Amino Acids in Feeding Responses of Adult Western Corn Rootworms (*Diabrotica virgifera virgifera*) to Corn (*Zea mays*) Pollen

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Received: 27 October 2006 / Accepted: 29 December 2006 /
Published online: 1 February 2007
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Abstract Adult beetles of *Diabrotica virgifera virgifera* LeConte (western corn rootworm) feed on pollen of *Zea mays* L. (corn) and other plant species. To identify *D. virgifera* feeding stimulants, beetle responses to mixtures of known and novel phagostimulants, presented at their naturally occurring concentrations in maize pollen, were compared to individual component responses applying the amount occurring in 0.2 mg of pollen per cellulose feeding disk. On a molar basis, three major sugars (fructose, glucose, and sucrose) were more prevalent in corn pollen buffer extract (CPE) than free amino acids. Western corn rootworm feeding was stimulated by the three sugars (28% disk consumption) and, to an even greater extent, by a mixture of 21 free amino acids (41% disk consumption). However, the combination of three sugars and 21 amino acids elicited a level of *D. virgifera* feeding (41% disk consumption) similar to that of the 21 amino acids alone. A novel maize pollen phagostimulant was purified from CPE by using solid-phase extraction followed by RP-HPLC. Based on its mass fragment pattern, two UV maxima (254 and 359 nm), and previous isolation from maize pollen, this phagostimulant is tentatively identified as isorhamnetin 3-*O*-neohesperidoside. This compound interacted additively with the mixture of three sugars and 21 amino acids, to produce 77% of the phagostimulation level of CPE. Therefore, a possible stimulatory mechanism for *D. virgifera* feeding on corn pollen has been elucidated.

Keywords Amino acids · Chrysomelidae · Diabrotica · Feeding stimulant interactions · Flavonol glycoside · Flavonoid · Maize · Phagostimulants · Pollen chemistry · Sugars

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Introduction

Pollen feeding by beetles is thought to be a primitive insect–plant association. Feeding on gymnosperm pollen by beetles predates the advent of angiosperms (Samuelson 1994), and angiosperm radiation has been associated with increase in beetle diversity (Farrell 1998). More than 184 angiosperm species are pollinated almost exclusively by beetles (Bernhardt 2000), but the pollen-feeding behavior of Coleoptera has been overshadowed by the greater incidence of this trait in the Hymenoptera.

There is quantitative and qualitative variability in the nutrients, including protein, amino acids, sugars, sterols, vitamins, and lipids, found in the pollen of different plant species (Stanley and Linskens, 1974). Chemical components of *Zea mays* L. (corn) pollen have been studied extensively. Sucrose is the predominant sugar and comprises 98% of the soluble carbohydrates and 5% of the pollen fresh weight (Hoekstra et al., 1989). The amount of fructose and glucose is inversely proportional to sucrose content and depends on the pollen storage conditions (Speranza et al., 1997). In sweet corn pollen, proline was the most abundant free amino acid, followed by serine and alanine (Stanley and Linskens, 1974; Hollister and Mullin, 1999). Flavones are absent from maize pollen, but ten flavonol glycosides, derived from kaempferol, quercetin, and isorhamnetin, have been reported (Ceska and Styles, 1984). Among these, quercetin 3,3′-*O*-diglucoside is the most abundant. Pollen is the corn tissue with the greatest concentration of sterols, with a profile dominated by 24(28)-methylene-sterols (Guo et al., 1995) and small amounts of castasterone and other brassinosteroids (Suzuki et al., 1986). Other biochemical components in *Zea mays* pollen have also been identified (Bianchi et al., 1990; Anaya et al., 1992; Radlowski et al., 1996; Bih et al., 1999).

Few studies have investigated the effects of host pollen chemical components on beetle feeding behavior. As one example, the Mexican beetle *Zygogramma bicolorata* Pallister (Chrysomelidae) feeds selectively on pollen of the weed *Parthenium hysterophorus* L. (Compositae), which contains parthenin, a sesquiterpene lactone feeding stimulant for this species (Jayanth et al., 1993). Phagostimulants for adult western corn rootworms (WCR), *Diabrotica virgifera virgifera* LeConte, from several pollen hosts have been investigated by using a thin-layer chromatography (TLC)-based feeding bioassay (Hollister and Mullin, 1999; Lin and Mullin, 1999). L-Alanine and L-serine are the most phagostimulatory amino acids for adult WCR (Hollister and Mullin, 1998; Kim and Mullin, 1998). Some lipids and midpolarity compounds from sunflower (*Helianthus annuus* L.) pollen that potentiate amino acid phagostimulation have also been identified (Lin and Mullin, 1999).

Adult WCR feed on pollen and other nutritious floral parts of host plants. Sweet corn and winter squash (*Cucurbita maxima* Duchesne) pollens are preferred. The essential role of pollen feeding in the reproductive and developmental success of many arthropods may be explained by the relatively high nutrient content, absence of harmful deterrents, and lack of fitness-reducing phytochemicals in this food compared to other plant tissues (Roulston and Cane, 2000). Elliot et al. (1990) demonstrated that the fecundity of maize pollen-fed female WCR was greater than that of beetles excluded from pollen. Maize pollen also enhanced development of other insect species (Ye-Ebiyo et al., 2000).

A structure–activity relationship study that used amino acids and derivatives suggested that multiple amino acid sites on beetle taste receptors interact to produce the net phagostimulatory input to the central nervous system (Kim and Mullin, 1998; Mullin and Kim, 2001). However, the feeding behavior of adult WCR on maize pollen was not explained by free amino acids and/or sugars only, suggesting that other phagostimulatory components are present. The objective of this study was to identify novel feeding stimulants in maize pollen and to examine the effects of phagostimulant combinations on WCR feeding responses.

Methods and Materials

Insects A nondiapausing laboratory strain of western corn rootworm (WCR) was purchased from French Agricultural Research, (Lamberton, MN, USA). Adult beetles were fed sliced store-bought acorn squash fruit and water *ad libitum* and were maintained at 25±1°C and a 16L:8D photoperiod in an M-31 Environmental Growth Chamber (Chagrin Falls, OH, USA).

Corn Pollen Extraction Corn pollen (sweet corn, *Zea mays* L. var. Bodacious) was extracted with 30 mM tripotassium citrate (pH 6.0) buffer as previously reported (Kim and Mullin, 2003).

Free Amino Acid Analysis Free amino acid (AA) analysis was carried out as described (Hollister and Mullin, 1999). The fraction containing AA after Dowex 50X8 cation-exchange chromatography (H^+ form) of CPE was derivatized with phenylisothiocyanate (PITC) and then analyzed by HPLC (Cohen and Strydom, 1988) with a Waters Novapak C_{18} reverse-phase column (4 μm, 3.9×150 mm, 60 Å) and a Waters 600E Multisolvent Delivery System with a 2487 Dual λ Absorbance Detector. The HPLC mobile phase consisted of: (A) 140 mM sodium acetate, with 0.5 ml triethylamine/liter, titrated to pH 6.35 with glacial acetic acid; and (B) 60% acetonitrile with a linear gradient of 2 to 50% eluent B in A over 35 min at 1.5 ml/min. The absorbance was measured at 254 nm.

Sugar Analysis The pollen buffer extract (CPE, 2 ml) was applied to a solid-phase extraction (SPE) tube (6 ml, Discovery DSC-18, Supelco), which was equilibrated with water before loading the sample. Nonbinding materials were eluted with water (6 ml) and then 10, 40, and 100% methanol (4 ml each) were used subsequently to wash off bound compounds. A water fraction containing sugars and free amino acids was evaporated to dryness with a Savant SC110 SpeedVac, whereas the three methanol fractions were dried using a Büchi Rotovapor (Brinkmann, Westbury, NY, USA) and later used for isolation of flavonoids. The dried water fraction was redissolved in 0.5 ml H_2O and applied to a Dowex 50X8 cation-exchange chromatography column (Hollister and Mullin, 1999) to separate sugars from free amino acids. Three sugar standards, fructose, glucose, and sucrose were processed in the same manner to evaluate their recovery rates. Isocratic separation of fructose, glucose, and sucrose by HPLC was achieved on a Cyclobond II (γ-cyclodextrin, 5 μm, 4.6×250 mm) column (Astec, Whippany, NJ, USA). The eluent was 90% acetonitrile at a flow rate of 2 ml/min and UV detection at 195 nm.

TLC Analysis The water fraction (SPE Water) containing sugars and amino acids after solid-phase extraction (SPE) as well as the corn pollen extract were also analyzed by TLC (LK5DF, 20×20 cm plate, 19 channels, Whatman) using standard sugars (fructose, glucose, and sucrose) and AA. For sugar analysis, the TLC plate was developed with ethyl acetate–MeOH–water (5:1.5:0.5) and the same developing solvent system with a different ratio (5:4:2) was used to separate free amino acids. After being air-dried, the plate was sprayed with naphthoresorcinol or ninhydrin and then heated for 5–10 min at 100°C to maximize sugar (Walters and Mullin, 1988) or amino acid (Hollister and Mullin, 1999) visualization, respectively.

Identification of Phagostimulants in the Corn Pollen Extract All phagostimulatory components bound by reverse-phase SPE of the corn pollen extract were eluted with 40% methanol (Fig. 1). This methanol-wash fraction (SPE MeOH) was analyzed by using reverse-phase HPLC (Novapak C_{18} column, 4 μm, 3.9×150 mm, 60 Å). The eluent system

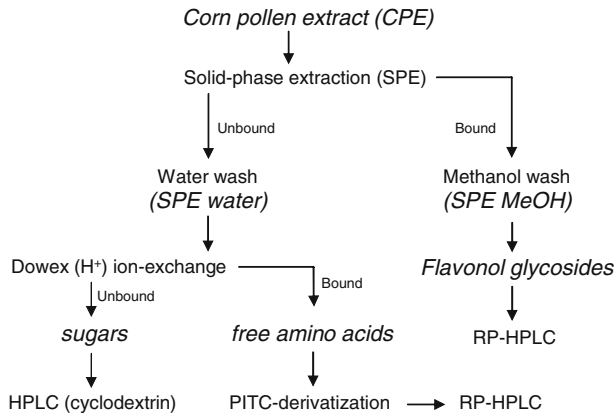


Fig. 1 Scheme for isolation of phagostimulants for adult western corn rootworm from corn pollen

consisted of (A) 75% methanol and (B) H_2O; going from 40 to 100% of eluent A in 15 min at 1.0 ml/min with a linear gradient. Absorbance was measured at both 254 and 359 nm to guide collection of peak fractions, which were then dried with a Rotavapor. Each fraction was dissolved in 20 mM sodium citrate buffer (pH 5.5) or 40% methanol for use in feeding bioassays. The UV–visible spectra of a purified bioactive sample were measured in methanol with a Waters 2487 Dual λ Absorbance Detector. One fraction out of 6 SPE MeOH fractions collected from HPLC showed a mild phagostimulatory effect on beetle feeding, and the phagostimulatory compound was identified with a Waters 2695 HPLC and detected with a Waters 2996 photodiode array detector and a Micromass Platform LC mass spectrometer (Waters, Bedford, MA, USA). A C_{18} HPLC column (LiChrospher RP-18, 250×4 mm, 5 μm endcapped, Varian, Lake Forest, CA, USA) was connected through a microsplitter valve (Upchurch Scientific, Apple Valley, MN, USA) to maintain a flow rate (200 μl/min) to ESI-MS. LC/ESI mass spectra were acquired by scanning the mass range from 100 to 700, and data acquisition and processing were controlled by using MassLynx v.3.5 software (Micromass, Manchester, UK). The eluent system consisted of (A) 90% acetonitrile with 0.05% formic acid and (B) H_2O with 0.05% formic acid. Column linear gradients for samples were: 0–5 min 40% A, 5–6 min 40–100% A, 6–7 min 100–40% A, and 7–8 min 40% A.

Feeding Bioassay Studies on phagostimulants were performed by using a no-choice bioassay (Hollister and Mullin, 1998). All feeding bioassays were performed by using postmated females and males aged between postemergence days 8 and 35 because mating occurred maximally between postemergence days 5 and 7 under our lab conditions. This reduced the possible interaction in a bioassay arena between randomly selected beetles. Beetles were usually used in consecutive bioassays with a 3- to 5-d interval (36- to 84-hr squash fruit feeding followed by 12-hr starvation, then 24-hr feeding bioassay). Beetles were deprived of food, but not water, for 12 hr before the bioassay. After starvation, two beetles (sex undetermined) were randomly placed into a 100×20-mm Petri dish containing one regenerated cellulose disk (26.5 mm^2, 1.0 μm pore size; Schleicher and Schuell, Daytona Beach, FL, USA) suspended in the middle of the dish on a minuten pin 2 mm above a paraffin layer covered with a moistened filter paper. Chemicals dissolved in an appropriate organic solvent, buffer or water (4 μl—this volume completely saturated a disk) were applied with a Hamilton repeating dispenser (Reno, NV, USA) to the disk. After a 24-hr feeding period, dry disk areas were determined

to the nearest 0.1 mm^2 with an Optomax V image analyzer (Analytical Measuring Systems, Saffron Walden, UK) and then subtracted from the mean disk area (26.5 mm^2) of 10 disks not offered to beetles, to give the area consumed. Consumption data are presented as a mean percentage of the disk area consumed per two beetles ± standard error of the mean (SEM) after 24 hr (water control=4.3±3.4%, N=24). At least eight replicates of each treatment were run for each of at least six independent feeding bioassays. Combined data were square-root transformed to improve normality and homogeneity of variance, and a one-way ANOVA was used to test for treatment effects followed by the Tukey–Kramer test using JMP 6 (SAS Institute, Cary, NC, USA).

Sample concentrations used in all feeding bioassays were approximated from that found in a 0.2-mg pollen equivalent. For example, the 21 amino acid sample contained proline (8.1), serine (2.8), alanine (1.2), γ-aminobutyric acid (GABA, 0.9), glutamine (0.8), aspartic acid (0.4), glutamic acid (0.4), arginine (0.2), threonine (0.2), and valine (0.2 nmol/disk) + 11 amino acids (asparagine, cysteine, glycine, histidine, isoleucine, leucine, lysine, methione, phenylalanine, tryptophan, and tyrosine, 0.08 nmol/disk each), and the three sugar samples consisted of fructose, glucose, and sucrose, at 37.7, 31.7, and 17.1 nmol/disk, respectively. All standard amino acids and sugars were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Results

Three major sugars, fructose, glucose, and sucrose, were more prevalent in the corn pollen buffer extract (CPE) than free amino acids (AA) on a molar basis (Table 1). Fructose and glucose were about twice as abundant as sucrose, and were the only monosaccharides and disaccharides detected. Proline dominated the AA, followed by serine, alanine, and GABA, which is consistent with previous findings by Hollister and Mullin (1999). The only significant difference in AA content of corn pollen between the present study and one by

Table 1 Sugar and free amino acid contents of the corn pollen buffer extract

Chemical	Content (nmol ± SEM/mg pollen equivalent)
SUGARS[a]	
Fructose	188.4±3.67
Glucose	158.5±2.91
Sucrose	85.6±0.71
AMINO ACIDS[a]	
Proline	40.6±0.55
Serine	14.0±0.09
Alanine	6.1±0.04
GABA	4.7±0.02
Glutamine	4.2±0.02
Aspartic acid	2.1±0.01
Glutamic acid	1.3±0.01

GABA γ-aminobutyric acid, *SEM* standard error of the mean

[a] Sugar (N=7) or free amino acid (N=5) analyses were performed on three independent extracts, and the seven dominant amino acids are shown.

Hollister and Mullin (1999) was that glutamine replaced asparagine as a major amino acid. To measure phagostimulatory activity, concentrations of standard chemicals or pollen extract fractions were fixed at approximately 0.2-mg pollen-equivalent per disk. This amount was dramatically lower than those used in two previous studies with adult WCR [3.6- and 5-mg pollen equivalent for Hollister and Mullin (1999) and Lin and Mullin (1999), respectively], but stimulated two beetles to consume 50–100% of a regenerated cellulose disk in 24 hr (J. Kim, personal observation). The isolation scheme to discover and analyze phagostimulatory compounds found in the corn pollen extract is depicted (Fig. 1).

The mixture of two known phagostimulatory amino acids [serine (2.8 nmol/disk) and alanine (1.2 nmol/disk), Hollister and Mullin, 1998; Kim and Mullin, 1998] or the 19-AA mixture lacking serine and alanine (12.18 nmol total/disk) stimulated 25 and 31% of disk consumption, respectively (Fig. 2). Because feeding responses were usually highly variable among individual bioassays, data from independent experiments were combined. Feeding response to the combined mixture of 21 AA (41%) was significantly greater than to the two-AA mixture (25%), but not to the 19-AA mixture (31%). Although the total molar concentration of the three sugars was much higher (86.5 nmol/disk) than that of the 21 AA (16.18 nmol/disk total), feeding response to the sugars (28%) was lower and similar to responses produced by the 2- or 19-AA mixtures. It is interesting to note that feeding response to the mixture of three sugars and 21 AA was not significantly different from either the 21 AA combination or the three-sugar mixture, alone. Moreover, the combined AA and sugars (39% disk consumption) only attained 57% of the phagostimulatory level (69% disk consumption) of the CPE (Fig. 3).

The free amino acids and sugars were present in the water fraction (SPE Water) after SPE, whereas the methanol fraction (SPE MeOH) contained weakly hydrophobic compounds such as flavonoids and other phenolics (Fig. 1), many of which absorb at 359 nm. Feeding bioassays on fractions collected from HPLC of SPE MeOH showed that only the sixth peak

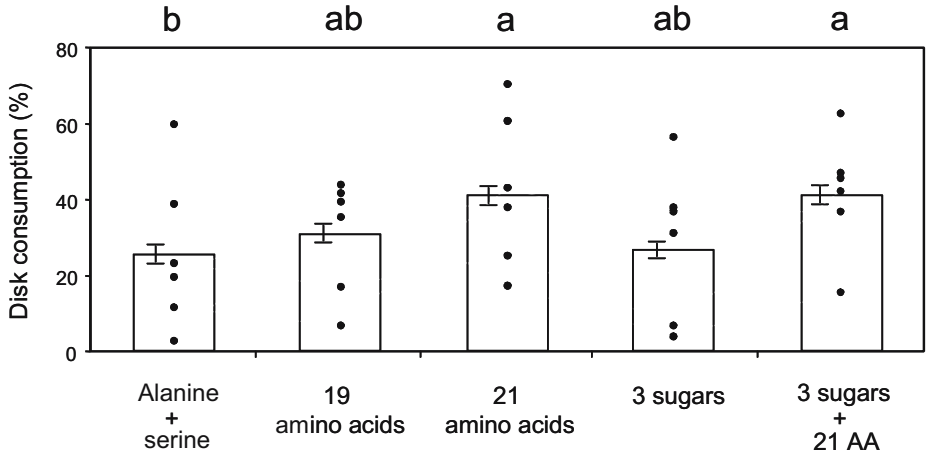


Fig. 2 Feeding responses (% disk consumption ± SEM) to amino acids, sugars and a mixture of the two. Twenty-one amino acids (AA) and three sugars were at approximately 0.2-mg pollen equivalent/disk. Each point indicates the mean values of six independent runs (eight replicates each) while the bars represent the mean of the combined data. Different letters represent significant differences by the Tukey–Kramer test ($P<0.05$) after a square-root transformation of values

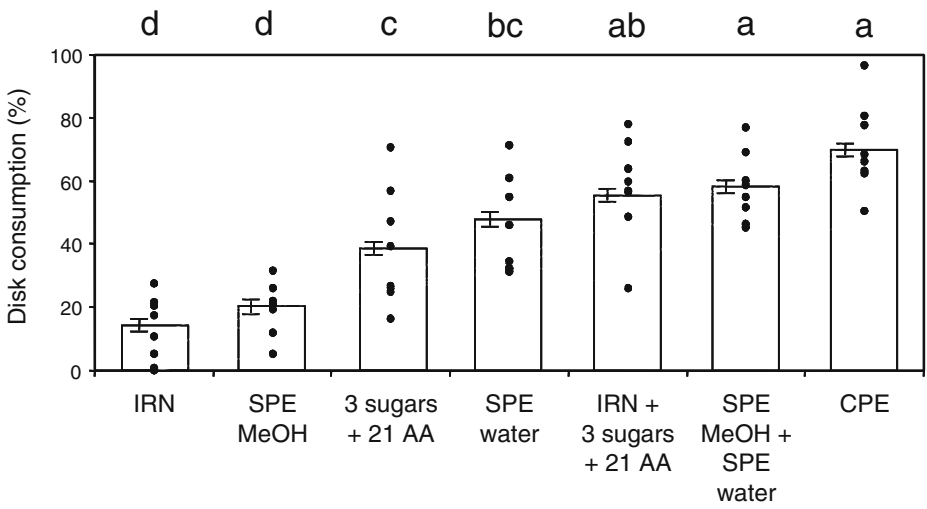
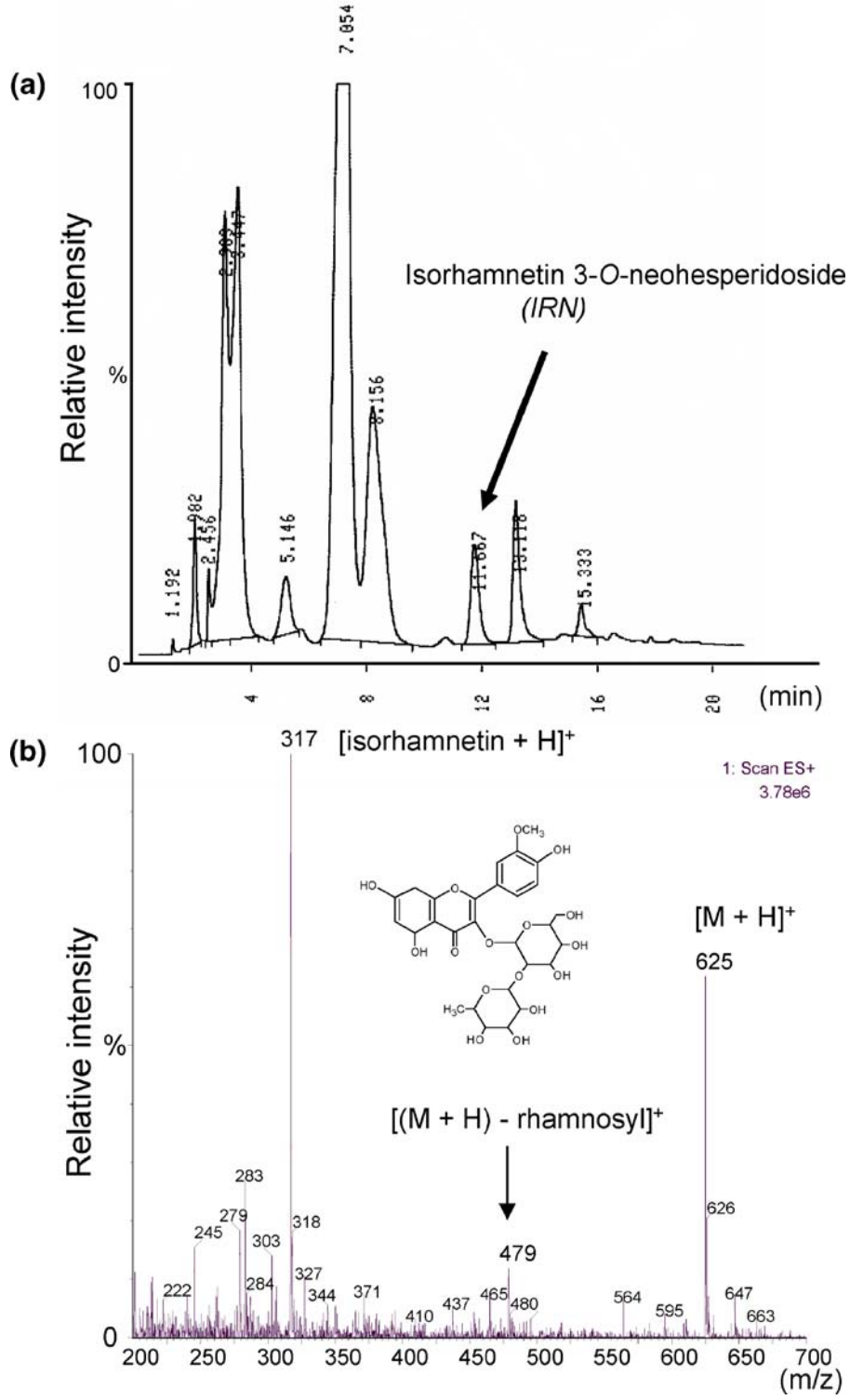


Fig. 3 Feeding responses (% disk consumption ± SEM) to phagostimulatory components in the corn pollen extract (CPE). The methanol (SPE MeOH) or water (SPE Water) fractions after solid-phase processing of CPE contained a flavonol diglycoside (IRN, see Fig. 4) or 21 amino acids and three sugars, respectively. All samples used in feeding bioassays were approximately at a 0.2 mg-corn pollen equivalent/disk. Each point indicates the mean values of eight independent runs (at least eight replicates each) while the bars represent the mean of the combined data. Different letters represent significant differences by the Tukey–Kramer test ($P<0.05$) after a square-root transformation of values

by area and in order of decreasing polarity at retention time 11.7 min (Fig. 4a) was slightly phagostimulatory (15% disk consumption), whereas other peaks did not stimulate beetle feeding (data not shown). This fraction also additively enhanced phagostimulation elicited by the mixture of three sugars and 21 AA (Fig. 3). The UV–visible spectrum of this purified fraction in methanol showed two major absorbance peaks at 254 and 359 nm (Mabry et al., 1970; Markham, 1982), and mass fragments were observed at *m*/*z* 479 $[(M + H)\text{-rhamnosyl}]^+$ and 317 $[\text{isorhamnetin} + H]^+$ (Fig. 4b). The absence of a *m*/*z* 463 $[(M + H)\text{-glucosyl}]^+$ fragment for a diglycosyl–isorhamnetin indicates the unknown is a monosubstituted rhamno-glucoside. Based on this UV, MS, and HPLC data in comparison with literature on known corn pollen and other floral flavonol glycosides (Ceska and Styles, 1984; Chaurasia and Wichtl, 1987; Nørbaek and Kondo, 1999), we tentatively identify this phagostimulant as isorhamnetin 3-*O*-neohesperidoside (IRN).

There was no significant difference in feeding responses between SPE MeOH and IRN, suggesting that IRN accounts for most of the phagostimulation elicited by the SPE MeOH fraction (Fig. 3). Likewise, feeding responses to the mixture of three sugars and 21 amino acids were not significantly different from SPE Water, which contained the free AA and sugars, suggesting that AA and sugars are responsible for most of the phagostimulation elicited by the SPE water fraction. Feeding response to the mixture of SPE MeOH and SPE water was significantly different from either SPE MeOH or SPE water, and beetle feeding was enhanced additively, which was not significantly different from feeding responses to CPE (Fig. 3). This additive feeding response was also observed in the case of IRN and 3 sugars + 21 AA. Feeding response to the mixture of IRN and three sugars + 21 AA was significantly different from either IRN or the three sugars + 21 AA individually.


(a)
100
%
0
Relative intensity
7.054
8.156
5.146
2.456
1.192
11.667
13.118
15.333
Isorhamnetin 3-O-neohesperidoside
(IRN)
4
8
12
16
20
(min)
(b)
100
%
0
Relative intensity
317 [isorhamnetin + H]+
1: Scan ES+
3.78e6
[M + H]+
625
[(M + H) - rhamnosyl]+
479
283
279
245
303
318
327
284
222
344
371
410
437
465
480
564
595
626
647
663
200
250
300
350
400
450
500
550
600
650
700
(m/z)

TLC was used to compare the amounts of sugars or amino acids in SPE or CPE after derivatization with standard spray reagents. Visual intensities of color reactions for sugars with naphthoresorcinol or AA with ninhydrin in SPE water or CPE on the TLC plate were similar to those of equivalent amounts of standard sugars or AA (J. Kim, personal observation).

Effects of phagostimulant mixtures on feeding responses were studied by comparing the observed feeding response (% disk consumption elicited by a mixture) with the expected feeding response (the sum of % disk consumption elicited by each component). Beetle feeding was enhanced additively in response to the combination of SPE MeOH with SPE Water (89% of the sum of feeding responses to each component) or the combination of IRN with the three sugars and 21 amino acids (98%) (Fig. 3). On the other hand, the observed feeding response to the mixture of 2 and 19 AA was much lower (73%) than the expected value. In addition, the mixture of three sugars and 21 AA was less than the expected value (59% of the sum of each component response) (Fig. 2).

Discussion

The molecular weight and fragment patterns (Fig. 4) of the unknown compound from SPE MeOH fraction of the corn pollen extract and its two UV maxima (Mabry et al., 1970; Bacon et al., 1975; Markham, 1982) suggest a monosubstituted flavonol diglycoside. Only isorhamnetin among the possible *O*-methylethers of quercetin has been reported in maize pollen, and no rutinosides (6-*O*-rhamnopyranosyl-β-D-glucopyranoside) or other rhamnosides have been detected in this maize organ (Ceska and Styles, 1984). This 3-*O*-substituted neohesperidoside (2-*O*-rhamnopyranosyl-β-D-glucopyranoside) of isorhamnetin has been found in flowers and pollens of other plants (Chaurasia and Wichtl, 1987; Nørbaek and Kondo, 1999), and is one of the active flavonol glycosides in cardioprotective typhae (cattail) pollen, the Chinese medicine pu huang (Zhou, 2004). Although ^{13}C-NMR would be required to rigorously distinguish this neohesperidoside from the respective rutinoside, we conclude that isorhamnetin 3-*O*-neohesperidoside (CAS# 55033-90-4), the fifth or sixth major flavonol glycoside, previously reported from maize pollen (Ceska and Styles, 1984), is the likely candidate for this rootworm phagostimulant.

Simmonds (2003) reviewed the dynamic roles of flavonoids as feeding and oviposition stimulants or deterrents in insect–plant interactions, particularly in reference to Lepidoptera. In Coleoptera, several flavonoids are known phagostimulants and antifeedants for various chrysomelid beetles (Matsuda, 1978; Nielsen et al., 1979). Lin and Mullin (1999) demonstrated that quercetin 3-*O*-β-glucoside extracted from sunflower pollen was mildly phagostimulatory to adult WCR. In the field situation, however, insects taste a complex mixture of both primary and secondary phytochemicals, which can act as feeding stimulants, deterrents, or neutral compounds. Nonetheless, few insect studies have investigated the feeding effects of phagostimulant mixtures, especially both nutrients and

◀**Fig. 4** (**a**) Reverse-phase HPLC analysis of a methanol-wash fraction after processing through a solid-phase extract (SPE MeOH) from the corn pollen buffer extract. Only one fraction (IRN) stimulated beetle feeding in an additive manner over that of a mixture of three sugars and 21 amino acids (see Fig. 3). Absorbance was measured at 359 nm. (**b**) LC-ESI-mass spectrum of the purified phagostimulatory fraction (IRN) from (A) indicates isorhamnetin-3-*O*-neohesperidoside

plant secondary metabolites, in comparison to the individual component responses at their naturally occurring concentrations.

All of the free amino acids present in maize pollen should be considered when evaluating their role in eliciting pollen feeding. Phagostimulation elicited by the 21 AA combination was significantly greater than for the binary blend of the primary phagostimulatory amino acids, serine, and alanine. Surprisingly, the remaining 19 AA had phagostimulatory activity similar to these latter two AA at corn pollen concentrations (Fig. 2). It is unclear how this 19 AA mixture elicited a moderate level of beetle feeding without containing a strong stimulatory amino acid (Kim and Mullin, 1998), although proline is mildly phagostimulatory (Hollister and Mullin, 1998).

Phagostimulatory interactions between three sugars and 21 free amino acids on beetle consumption were less than additive (Fig. 2), indicating the probability of a competitive interaction at common taste receptor sites. By contrast, IRN enhanced reference-feeding responses in an additive manner when combined with the phagostimulatory mixture of three sugars and 21 AA (Fig. 3). It is interesting to note that a high concentration (up to 1-mg pollen equivalent) of IRN did not further enhance beetle feeding (data not shown). This indicates that IRN is not a strong phagostimulant by itself, but appears to modulate beetle feeding response when combined with other phagostimulatory compounds. For example, a combination of flavonol glucosides and sinigrin elicited a significant feeding preference in diamondback moth, *Plutella xylostella* L., for the mixture over the glucosinolate alone (van Loon et al., 2002). Flavonoids are known to be "biting" and "swallowing" factors for *Bombyx mori* (Hamamura, 1970), and their potential role in these mechanisms for interactions of phagostimulants at beetle taste receptors or their postingestive effects on beetle fitness for WCR need further evaluation.

The opposite relative amounts of glutamine and asparagine between the present study where glutamine is higher and Hollister and Mullin (1999) may reflect differences in extraction efficacy (buffer in the present study vs. aqueous methanol), or changes in pollen composition or extractability of free amino acids due to storage periods up to 6 years at −80°C. Sucrose has been reported as the dominant sugar in corn pollen (Hoekstra et al., 1989), but fructose and glucose were more prevalent than sucrose in the CPE used in this study. Degradation during buffer extraction or pollen storage may account for these discrepancies. Nevertheless, a buffer extract from recently collected corn pollen (e.g., var. 'Jackpot' in 2001) elicited the same level of phagostimulation as did the CPE used in this study (data not shown), although its contents of sugars and AA were not examined.

Whereas lipophilic compounds in corn pollen failed to stimulate or deter beetle feeding (J. Kim, personal observation), hydrophilic components, especially free amino acids and sugars, explained about 57% of total phagostimulation elicited by CPE (Fig. 3). Isorhamnetin 3-*O*-neohesperidoside, in addition, served as a costimulant for the combined three sugars and 21 AA explaining about 77% of the CPE consumption (Fig. 3). However, unknown compounds interacting with known phagostimulants may account for phagostimulatory factors yet to be discovered in maize pollen. Future studies are needed to address the mechanisms by which primary chemical stimuli for pollen feeding interact, and how these key behavioral factors for beetle feeding may be used in design of control tactics for adult WCR.

Acknowledgments We thank Georg Jander for helpful comments and use of the LC/MS at the Boyce Thompson Institute for Plant Research and Alan Renwick for comments on the manuscript. This work was supported, in part, by USDA grant #95-37302-1807 and Atlantic Philanthropies.

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J Chem Ecol (2007) 33:513–524
DOI 10.1007/s10886-006-9232-7

Sequestration of Furostanol Saponins by *Monophadnus* Sawfly Larvae

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Received: 28 June 2006 / Revised: 3 October 2006 / Accepted: 27 November 2006 /
Published online: 25 January 2007
© Springer Science + Business Media, LLC 2007

Abstract Sawfly larvae of the tribe Phymatocerini (Hymenoptera: Tenthredinidae), which are specialized on toxic plants in the orders Liliales and Ranunculales, exude a droplet of deterrent hemolymph upon attack by a predator. We investigated whether secondary plant metabolites from Ranunculaceae leaves are sequestered by phymatocerine *Monophadnus* species, i.e., *Monophadnus alpicola* feeding upon *Pulsatilla alpina* and *Monophadnus monticola* feeding upon *Ranunculus lanuginosus.* Moreover, two undescribed *Monophadnus* species were studied: species A collected from *Helleborus foetidus* and species B collected from *Helleborus viridis*. Comparative high-performance liquid chromatographic–photodiode array detection–electrospray ionization–mass spectrometric analyses of plant leaf and insect hemolymph extracts revealed the presence of furostanol saponins in all samples. Larvae of species A and B actively sequestered (25*R*)-26-[(α-L-rhamnopyranosyl) oxy]-22α-methoxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→3)-*O*-[6-acetyl-β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranoside (compound **1**). This compound occurred at a 65- to 200-fold higher concentration in the hemolymph of the two species (1.6 and 17.5 μmol/g FW, respectively) than in their host plant (0.008 and 0.268 μmol/g FW, respectively). In *M. monticola*, compound **1** was found at a concentration (1.2 μmol/g FW) similar to that in the host plant (1.36 μmol/g FW). The compound could not be detected consistently in *M. alpicola* larvae where, however, a related saponin may be present. Additional furostanol saponins were found in *H. foetidus* and *H. viridis*, but not in the two *Monophadnus* species

Dedicated to the memory of Professor Ivano Morelli (1940–2005)

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feeding on them, indicating that sequestration of compound **1** is a highly specific process. In laboratory bioassays, crude hemolymph of three *Monophadnus* species showed a significant feeding deterrent activity against a potential predator, *Myrmica rubra* ant workers. Isolated furostanol saponins were also active against the ants, at a concentration range similar to that found in the hemolymph. Thus, these compounds seem to play a major role for chemical defense of *Monophadnus* larvae, although other plant secondary metabolites (glycosylated ecdysteroids) were also detected in their hemolymph. Physiological and ecological implications of the sequestered furostanol saponins are discussed.

Keywords *Monophadnus* sawfly larvae · Ranunculaceae · *Helleborus* · *Ranunculus* · *Pulsatilla* · Antipredator defense · Easy bleeding · Sequestration · Hemolymph · Furostanol saponins

Introduction

Specialization on plant species containing secondary metabolites with broad-range toxicity offers herbivores the opportunity to sequester these metabolites and to use them for their own defense (Duffey 1980). Sequestration of plant-derived secondary metabolites has been repeatedly described from Lepidoptera and Coleoptera species, and in a few cases from other insect orders such as the Orthoptera and Hymenoptera (Laurent et al., 2005).

Tenthredinid sawfly (Hymenoptera) species of different subfamilies are able to sequester plant secondary metabolites in larval hemolymph (Bowers et al. 1993; Schaffner et al., 1994; Müller et al. 2001). Laboratory and field experiments indicate that larval hemolymph from these sawfly species is highly deterrent to different groups of predators (Schaffner et al., 1994; Müller and Brakefield, 2003). Boevé and Schaffner (2003) observed that larvae with high hemolymph deterrence activity towards *Myrmica rubra* L. exhibit "easy bleeding", i.e., they possess an integument with such a low resistance that even slight mechanical damage provokes release of hemolymph. Sawflies that do not possess a deterrent larval hemolymph have an integument that is significantly more resistant to mechanical damage. Based on these observations, Boevé and Schaffner (2003) hypothesized that there is a functional link between larval integument resistance and hemolymph deterrence, jointly acting as a chemical defense strategy.

Sawflies of the closely related genera *Rhadinoceraea*, *Phymatocera*, and *Monophadnus* (Tenthredinidae, Phymatocerini) display a particularly low larval integumental resistance (Boevé and Schaffner, 2003). This implies that many if not all phymatocerine sawflies are able either to sequester harmful secondary plant metabolites from their host plant or to synthesize deterrents de novo, and to store them in their hemolymph. So far, sequestration of plant secondary metabolites has been found in *Rhadinoceraea nodicornis*, which takes up and stores steroid alkaloids from *Veratrum album* in the larval hemolymph (Schaffner et al., 1994). Sawflies of the genera *Rhadinoceraea* and *Phymatocera* feed on Liliales and Asparagales, while species of the genus *Monophadnus* live on Ranunculaceae (Liston 1995). The corresponding order of Ranunculales is distantly related to Liliales and Asparagales, belonging to the Di- and Monocotyledones, respectively. We suppose the diverging host specialization is linked to chemical similarities between the two plant taxa. Our hypothesis is that sawflies of the taxon Phymatocerini, which share the ability to bleed easily, also share the ability to sequester plant compounds.

Here, we investigated whether the sawflies *Monophadnus* species A (field host plant: *Helleborus foetidus* L.), species B (*Helleborus viridis* L.), *M. monticola* (Hartig) (*Ranunculus lanuginosus* L.), and *M. alpicola* Benson (*Pulsatilla alpina* L.) sequester plant secondary metabolites in their defensive hemolymph. The work was primarily devoted to furostanol saponins, since: (1) such steroid saponins occur in Ranunculaceae as well as in Liliales and Asparagales (Wissner and Kating, 1974; Hardman and Benjamin, 1976; Tschesche et al., 1984); (2) the closely related steroid alkaloids occur in Liliales and are sequestered by at least one Phymatocerini species (see above); (3) steroidal compounds possess well-known deleterious effects on animals (Teuscher and Lindequist, 1987; Harmatha 2000).

Material and Methods

Insect and Plant Samples

Sawfly larvae and leaves of their host plants were collected in different regions of Switzerland: *Monophadnus* species A on *H. foetidus* from the Jura mountains (Kantons Jura and Baselland), *Monophadnus* species B on *H. viridis* from south of the Alps (Kanton Ticino), *M. alpicola* on *Pulsatilla alpina* from the Bernese Oberland (Kt. Bern) and alpine regions of Kanton Wallis, and *M. monticola* on *Ranunculus lanuginosus* from the Jura mountains (Kt. Solothurn), the Bernese Oberland (Kt. Bern), and Kanton Wallis. Reference specimens of all species have been deposited in the collections of the Deutsches Entomologisches Institut, ZALF e.V. (Müncheberg, Germany).

Sawfly larvae were maintained in the laboratory on leaves of their host plants. Hemolymph droplets were collected in glass capillaries after gently piercing the larval integument with forceps. The hemolymph was accumulated in ethanol and stored at −20°C in the dark. Each ethanolic extract was sonicated in an ice bath for 15 min and then vortexed for 1 min. After centrifugation (5 min, 4,000 rpm), the supernatant was transferred to a new vial, and the precipitate was reextracted twice with methanol under the same conditions. The combined supernatants were dried under N_2, and redissolved in methanol up to a concentration of 1 to 3 mg/ml.

For quantitative analyses, hemolymph was obtained from 23 larvae of *Monophadnus* species B, five species A, six *M. alpicola*, and 10 *M. monticola*. A total of 203, 39, 103, and 69 μl of hemolymph was collected from these species, respectively. These four samples of hemolymph yielded 9.6 mg of dry extract (from which 4.8 mg were soluble in methanol), 3.5 (2.7), 9.9 (5.5), and 14.8 (5.4), respectively.

Full-grown leaves of *H. viridis*, *H. foetidus*, *P. alpina*, and *R. lanuginosus* were collected in the same areas as the larvae (see above). The fresh plant material was chopped and directly extracted in methanol at room temperature. After concentration under reduced pressure ($T<$ 40°C), the crude extract was partitioned with *n*-hexane, chloroform, *n*-butanol, and distilled water to obtain four corresponding fractions. After removing the solvent in a rotavapor ($T<40$°C), each fraction was freeze-dried, and the residue was dissolved in methanol, centrifuged, and the clear supernatant used for analyses (see Qualitative and Quantitative Analyses section).

Chemicals

By preliminary comparative LC-UV-MS analyses of each hemolymph sample and of several plant extracts, saponins were detected regularly in the hemolymph as well as in the

butanolic plant fraction. The isolation of sufficient quantities of purified saponins allowed us to run further analyses in specific conditions.

A standard of (25*R*)-26-[(α-L-rhamnopyranosyl)oxy]-22α-methoxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranoside (compound **2**; Fig. 1) was obtained as described in Braca et al. (2004).

Isolation of Compound **1**

Fresh leaves of *H. viridis* (180 g) were extracted until exhaustion with methanol at room temperature. The extract was evaporated to give a residue (9 g) that was subsequently partitioned between *n*-hexane, chloroform, *n*-butanol, and water, yielding fractions in the proportions of 21, 1, 20, and 58%, respectively. A portion of the *n*-butanol fraction (1.8 g) was chromatographed on Sephadex LH-20 column (100×5 cm) using methanol as eluent. Fractions of 8 ml were collected and grouped into 13 major fractions according to their features on thin layer chromatography (TLC) performed on silica 60 F_{254} gel-coated glass sheets with *n*-butanol–acetic acid–water (60:15:25) and chloroform–methanol–water (40:9:1). Fractions 19–21 of the Sephadex LH-20 column were further purified by preparative HPLC (flow rate 2.0 ml min^{-1}) with methanol–H_2O (6.5:3.5) to give compound **1** (t_R=15 min, 5 mg). The preparative HPLC system consisted of a Shimadzu LC-8A series pumping system equipped with a Shimadzu injector, a semipreparative Waters μ-Bondapak C_{18} column (30 cm×7.8 mm i.d.), and a Waters R401 refractive index detector. ^1H and ^{13}C NMR experiments, recorded in CD_3OD, were performed on a Bruker DRX-600 NMR spectrometer using the UXNMR software package. The spectra obtained were in accordance with those described by Braca et al. (2004) for (25*R*)-26-[(α-L-rhamnopyranosyl)oxy]-22α-methoxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→3)-*O*-

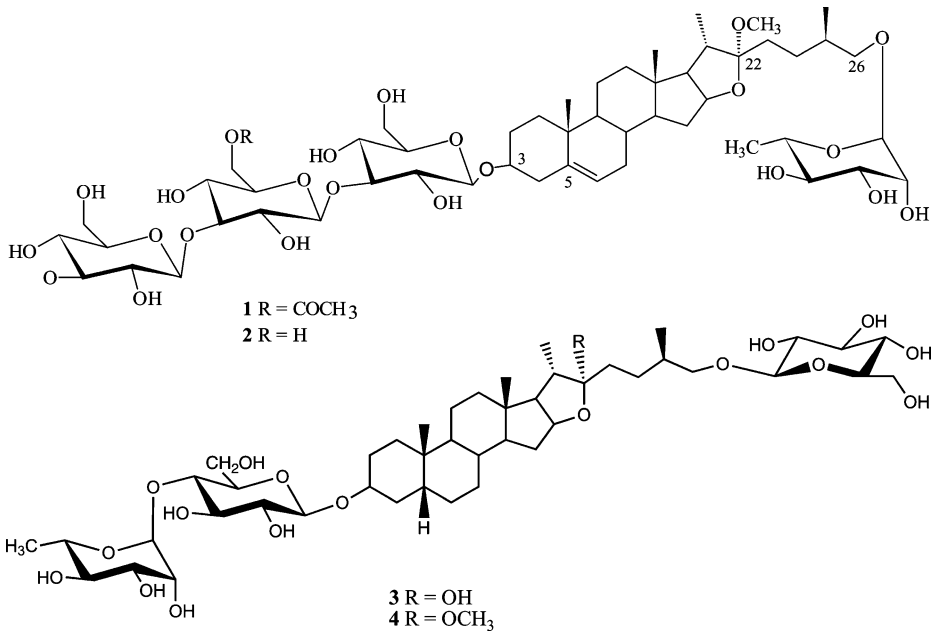


Fig. 1 Structures of furostanol saponins isolated from *Helleborus* species

[6-acetyl-β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranoside (Fig. 1), isolated from *H. viridis* collected at Alpe del Vicerè (Como Province, Italy).

Isolation of Compounds **3** and **4**

H. foetidus leaves (173 g FW) were extracted until exhaustion with methanol at room temperature and concentrated under vacuum ($T<40°C$). The residue (9 g) was redissolved in water and subsequently partitioned between *n*-hexane, chloroform, *n*-butanol, and water, yielding fractions in the proportions of 11, 0.5, 59, and 33%, respectively. The *n*-butanolic fraction (1.69 g) was chromatographed on a Sephadex LH-20 column (100×5 cm) using methanol as eluent. Fractions of 8 ml were collected and grouped into 13 major fractions by their TLC features as described above. The fourth (75 mg) of these fractions was further purified by preparative HPLC (see above) eluting with methanol–water (6.5:3.5, flow rate 2 ml min^{-1}), and the peak at $t_R=20$ min was collected (7.5 mg). The MS spectrum was in agreement with (25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxy-5β-furostan-3-β-yl *O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside, as described by Liang et al. (2002). This compound (i.e., compound **3** that contains a C-22 hydroxy group) was mixed with the C-22 methoxyl form (compound **4**; see Fig. 1).

Qualitative and Quantitative Analyses

High-performance liquid chromatographic–photodiode array detection–electrospray ionization–mass spectrometric (HPLC–UV/PDA–ESI/MS) analyses were performed by using a Surveyor LC pump, a Surveyor Autosampler, coupled to a Surveyor PDA detector and LCQ Advantage ion trap mass spectrometer equipped with Xcalibur 3.1 software (Thermo Finnigan, San Jose, CA, USA). Analyses were performed with a μ-Bondapak C_{18} column, 30 cm×3.9 mm i.d. (Waters Corporation, Milford, MD, USA). We followed the step gradient described by de Combarieu et al. (2002) using as solvents 0.01% aqueous formic acid (A) and acetonitrile (B): 0–20 min, 85–75% A, 15–25% B; 20–40 min, 75–55% A, 25–45% B; 40–55 min, 55–20% A, 45–80% B; 55–65 min, isocratic 20% A, 80% B. Flow rate was 1.0 ml/min. The column was at 23°C. UV channels were set at 246 and 200 nm.

ESI–mass spectrometer conditions were optimized to achieve maximum sensitivity for compounds **1** and **3**: positive ion mode, source voltage 4.5 kV, sheath gas (N_2) at flow rate 60 au, curtain gas (N_2) at 9.00 au, source current 80 A, capillary voltage 3 V, and capillary temperature 280°C. Full scan spectra were obtained in the positive ion mode, with a scan time of 1 sec, and from *m*/*z* 250 to 1,400. Ion trap conditions: acquisition in automatic gain control with a max-inject time of 200 ms. For the MS^2 analyses the most intense molecular ions were isolated with a width of 1 *m*/*z* unit and fragmented by using an activation amplitude of 25%.

To assess saponin concentration in plant and hemolymph samples, aliquots of four appropriate concentrations of the isolated saponin **1** and of the saponin mixture consisting of compounds **3** and **4** were injected onto the analytical system, eluted, and monitored with the ESI/MS detector under the conditions detailed above. Injections were performed in triplicates for plant samples and in duplicates for hemolymph samples, and peak areas were integrated and related to the amount of injected external standard. Linear regressions, interpolations, and Student–Newman–Keuls tests were done with the GraphPad Prism v.3 software (San Diego, CA, USA). Calibration curves and quantitative analyses of saponins in plant and hemolymph samples were replicated at three different days.

Feeding Deterrent Effect on Ants

Crude hemolymph was extracted in ethanol, filtered on celite powder, the solvent evaporated under a stream of N_2, and the dried extract then redissolved in sucrose 0.1 M. Two samples of purified saponins were also used. A sample of compound **1** (95% purity by HPLC–ESI/MS) contained a small amount of compound **2**, while a sample of compound **3** was inevitably in equilibrium with compound **4** (see above) in a ratio 2 to 1. The saponin samples were dissolved first in ethanol, then in sucrose 0.1 M (in volumes of 5 and 95%, respectively) to ensure good saponin dissolution. Preliminary tests comparing sucrose 0.1 M with ethanol in sucrose 0.1 M (1:19) gave no difference in feeding by *M. rubra* (see below).

The deterrent activity of the sawfly hemolymph and the purified steroidal saponins was assessed by the following bioassay. Forty *M. rubra* workers were placed in a Petri dish (9.5 cm diameter), in which they were offered a choice between 50 µl control solution (sucrose 0.1 M) and 50 µl of a test solution (12 replications). The number of ants feeding on each solution was recorded after 5 min. The test solution was sucrose 0.1 M containing 8 mg dry hemolymph or 0.8 mg purified plant secondary metabolite per milliliter solution. Eight-milligram dry hemolymph per milliliter solution was used as start concentration in previous works (Schaffner et al., 1994; Boevé and Schaffner, 2003; Boevé and Müller, 2005). Tenfold dilutions were then tested in the same way. The results were analyzed with the Wilcoxon matched-pairs signed-ranks test.

Results

Comparative analyses of sawfly hemolymph and fractions of their host plant leaves revealed that compound **1** was consistently detected in the following sawfly larvae: *Monophadnus* species A, species B, and *M. monticola*. Furthermore, the butanolic fraction of the plant *H. viridis* (Figs. 2 and 3) contained compound **1**. Compound **2**, that is the deacetylated form of compound **1**, was only detected in *H. viridis*, and compounds **3** and **4** were only present in extracts from *H. foetidus* (Fig. 3).

Compound **1** was also detected, but inconsistently, in *M. alpicola* and *P. alpina*. Furthermore, a peak (compound **5**) was detected in *M. alpicola* and *P. alpina* that showed a mass spectrum with a pseudomolecular peak at *m*/*z* 1,268 yielding a fragment at *m*/*z* 1,105.5 (loss of 162 Da) in a MS^2 experiment (see Table 1). This suggests that the structure of this compound (**5**) may correspond to compound **1** with an additional hexose moiety.

The response of the ESI/MS detector to different concentrations of compound **1** was linear in the range 0.01 to 0.25 mg/ml (r=0.999), and to those of compounds **3** and **4** (in a ratio 2:1) in the range 0.5 to 0.125 mg/ml (r=0.999). The resulting concentrations of the compounds in the hemolymph of *Monophadnus* species A, species B, and *M. monticola* as well as in their host plants are given in Table 2. Compound **1** was approximately 60- to 200-fold more concentrated in the hemolymph of *Monophadnus* species A and species B than in their respective host plants, indicating a particularly efficient mode of sequestration of compound **1** by these two sawfly species.

The larval hemolymph of *Monophadnus* species A, species B, and *M. monticola* was highly deterrent to ants at 8 mg DW per milliliter of solution (Table 3). At this concentration, no or almost no ants were feeding on the test solution. For these three species, a significant difference in the distribution of ants on test and control solutions was

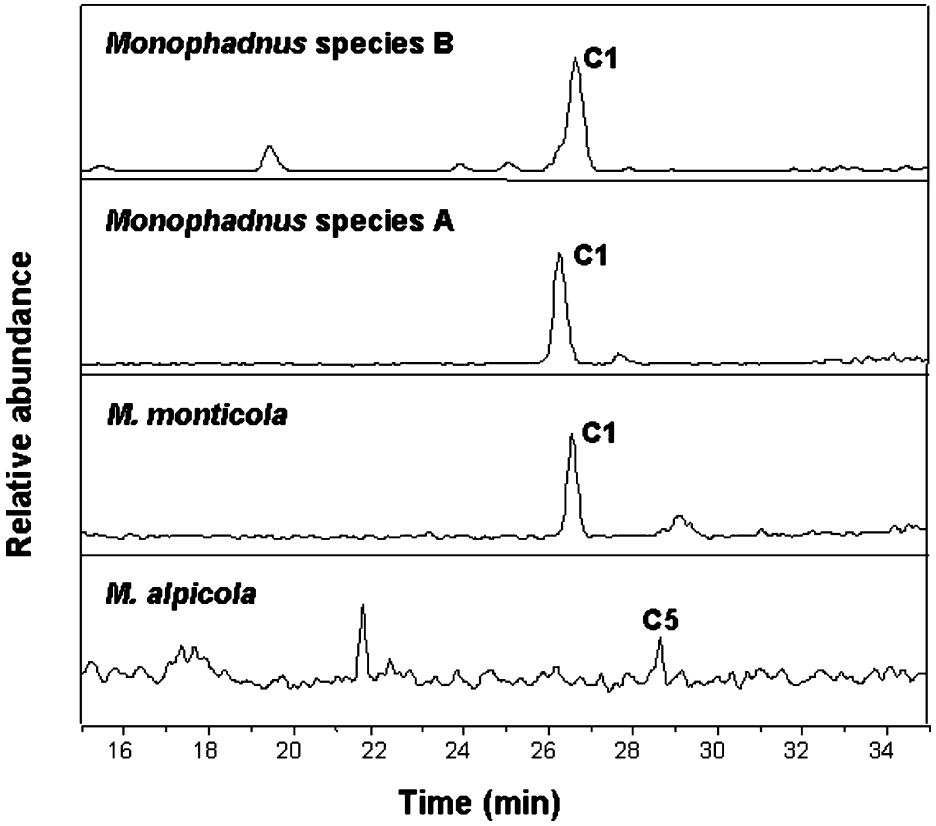


Fig. 2 HPLC–ESI/MS base peak chromatograms of hemolymph extracts from *Monophadnus* larvae, showing the occurrence of the furostanol saponins, compounds **1** and **5** (C1, C5)

lost at a concentration of 0.08 mg hemolymph DW per milliliter of solution. The tested furostanol saponins proved to be deterrent as well, up to this concentration, and compounds **3** and **4** showed a slightly higher deterrence than compounds **1** and **2** (Table 3).

Discussion

Among the furostanol saponins detected, compound **1** was the dominant one stored in the hemolymph of three *Monophadnus* species: *Monophadnus* species A, species B, and *M. monticola*. Among the host plants, *H. viridis* was the only one where compound **1** was a major steroidal saponin, while *H. foetidus* had mostly compounds **3** and **4**. Thus, it is likely that compound **1** is selectively sequestered by *Monophadnus* species A. It remains to be shown how furostanol saponins pass through the gut wall and how they are stored in the hemolymph, but it is likely that their uptake relies on an active transport and/or an efficient storage system, as proposed by several authors in other cases of sequestration (Duffey 1980; Bowers 1992).

In the plant material, compounds **1** and **4** are supposed to possess originally a hydroxyl group at C-22. This position is easily methoxylated under exposure to methanol (Liang

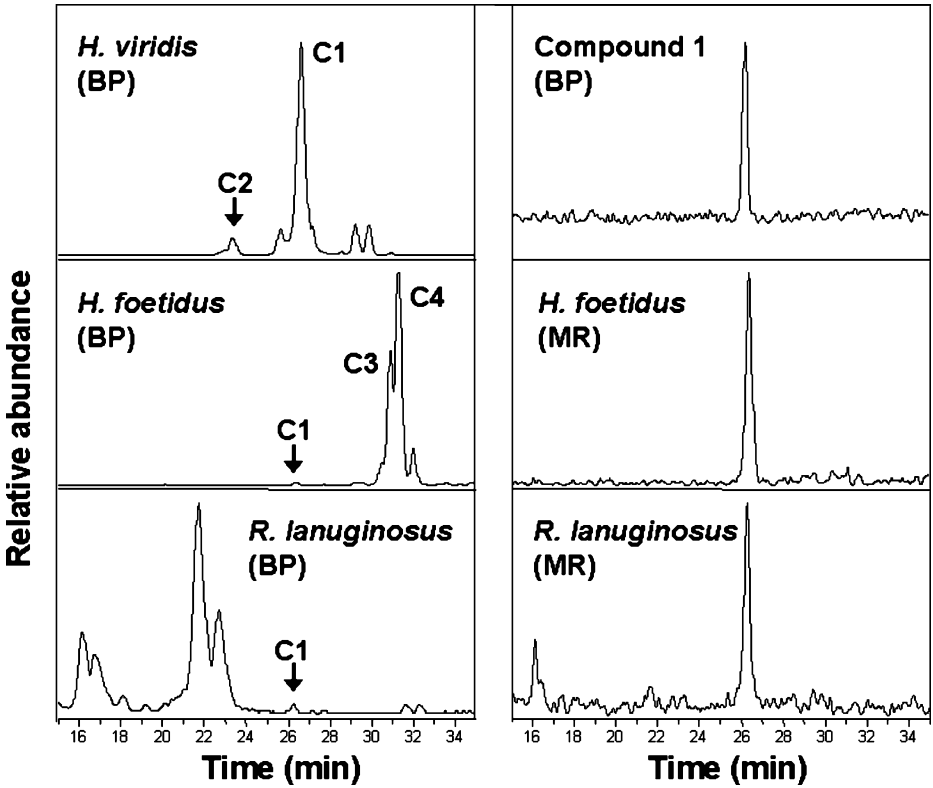


Fig. 3 HPLC–ESI/MS chromatograms of *n*-butanolic extracts from Ranunculaceae leaves, showing the occurrence of several furostanol saponins, compounds **1** to **4** (C1, C2, C3, and C4), and of isolated compound **1**. Base peak (BP) and mass range (MR; with m/z=1,105.5) chromatograms are shown

et al., 2002). Thus, different times of exposition to this solvent, during the extraction and subsequent isolation procedures, may have caused different methoxylation levels of the saponins present in the samples of *H. viridis* and *H. foetidus*. The same applies for the insect samples, which were also extracted in methanol, and for samples subjected HPLC–ESI/MS where methanol was used as well.

Triterpenoid saponins are part of the chemical defense in a number of leaf beetles (Termonia et al., 2002; Gillespie et al., 2003), but, to our knowledge, the results presented here provide the first evidence for sequestration of steroidal saponins into the larval body of insect herbivores. Criocerine leaf beetles incorporate steroidal saponins in fecal shields to enhance their defense (Morton and Vencl, 1998; Vencl et al., 1999). It is interesting to note that steroidal alkaloids, which are sequestered by the sawfly *Rhadinoceraea nodicornis* (Schaffner et al., 1994), have also been found in these criocerine fecal shields (Vencl et al., 1999). This suggests that the criocerine leaf beetles and Phymatocerini sawflies have evolved different ways to exploit the same group of plant secondary metabolites for their own defense.

Steroidal saponins are known to have deterrent and toxic effects on a broad range of invertebrates (Applebaum and Birk, 1979; Harmatha 2000). They act by forming an insoluble complex with sterols, such as cholesterol, which results in the breakdown of

Table 1 Chromatographic and spectral data of identified compounds from sawfly and plant samples

Rt	MW	ESI–MS^+ ions (*m*/*z*)	Compound	Detected in
26.3	1,120	1,105.5 $[M–CH_3+H]^+$	(25*R*)-26-[(α-L-rhamnopyranosyl) oxy]-22α-methoxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→3)-*O*-[6-acetyl-β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranoside (compound **1**)	*M.* species A, *M.* species B, *M. monticola*, *H. foetidus*, *H. viridis*, *R. lanuginosus*
22.8	1,078	1,064 $[M–CH_3+H]^+$	(25*R*)-26-[(α-L-rhamnopyranosyl) oxy]-22α-methoxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranoside (compound **2**)	*H. viridis*
30.9	904	887 $[M–H_2O+H]^+$	(25*R*)-26-[(β-D-glucopyranosyl) oxy]-22α-hydroxy-5β-furostan-3-β-yl *O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (compound **3**)	*H. foetidus*
31.2	918	904 $[M–CH_3+H]^+$	(25*R*)-26-[(β-D-glucopyranosyl) oxy]-22α-methoxy-5-β-furostan-3-β-yl *O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside (compound **4**)	*H. foetidus*
28.8	1,282[a]	1,268 (BP); (MS^2) 1,105.5. Proposed fragmentation: 1,268 $[M–CH_3+H]^+$,1,105.5 $[M–CH_3\text{-Hex}+H]^+$	Compound **1** + hexose (compound **5**) (?)	*M. alpicola*, *P. alpina*

BP Base peak

[a] Proposed molecular weight (Hex: 162 Da; Deohex: 146 Da)

sterol-based physiological processes such as molt or growth. In contrast to the criocerines, *Monophadnus* larvae must take up the steroidal saponins through the gut wall to accumulate them in their body, where it may directly interfere with these processes. It remains to be shown how *Monophadnus* larvae prevent autotoxicity by the sequestered steroidal saponins.

Sequestration of steroidal saponins appears to protect the larvae of all *Monophadnus* species studied from attack by ants. Predatory insects with biting–chewing mandibles constitute the type of predators that are most effectively deterred by the hemolymph released through woundings of the easily damaged integument of some sawfly larvae, including the *Monophadnus* species (Boevé and Schaffner, 2003; Müller and Brakefield, 2003; Boevé and Müller, 2005). In the field, workers of the ant *Lasius niger* L. retreated immediately after biting into a larva of *Monophadnus* species B in which they made contact with its hemolymph; the ants were then observed cleaning their mandibles for several minutes (Barker, personal observation). In our bioassays, the sequestered steroidal saponins tested as isolated, purified compounds proved to be highly deterrent to *M. rubra* ants (Table 3). The saponins were still active at a concentration of 0.08 mg saponins per milliliter solution (i.e., 0.07 μmol compound **1** per gram of FW solution), which is less than

Table 2 Concentration of furostanol saponins in larval hemolymph of *Monophadnus* species and leaves of their host plants

Species	Compound **1**	Compounds **3** and **4**
Monophadnus species A	17.5±3.5	–
H. foetidus	0.268±0.051	2.4±0.5
Monophadnus species B	1.6±0.3	–
H. viridis	0.0079±0.0008	–
M. monticola	1.2±0.4	–
R. lanuginosus	1.36±0.11	–

All values expressed as mean±SD of micromole compound per gram of FW hemolymph or leaves.

Negative sign (−) Not detected

the concentrations of compound **1** (at least 1.2 μmol/g FW) found in the hemolymph. Thus, it is likely that the chemical defense of the studied sawfly larvae is primarily based on the sequestration of steroidal saponins into the hemolymph. Besides the furostanol saponins, however, glycosylated ecdysteroids were detected in the hemolymph of *Monophadnus* species B. The hemolymph contained polypodine B glycoside and 20-hydroxyecdysone glycoside, whereas leaves of its host plant, *H. viridis*, contained their aglycones, polypodine B and 20-hydroxyecdysone (Prieto, unpublished data). Ongoing research aims to quantify their concentrations in larvae and host plant, and to determine their activity as feeding deterrents.

Steroidal saponins have been found in a variety of monocotyledonous plant families (Evans 1989). In contrast, Ranunculaceae constitute one of the few dicotyledonous families from which steroidal saponins have been described (Wissner and Kating, 1974; Petricic

Table 3 Feeding deterrent activity of *Monophadnus* larval hemolymph and of saponins against *M. rubra* ant workers

Test objects	Concentration (mg/ml solution)			
	8	0.8	0.08	0.008
Monophadnus species A	98*	42*[c]	21	–
	(82)	(79)	(109)	
Monophadnus species B	100*	62*[c]	−2	–
	(137)	(100)	(127)	
M. monticola	100*	48*[c]	11	–
	(55)	(77)	(94)	
Compounds **1** and **2**[a]	–	89*	25*	−2
		(114)	(114)	(217)
Compounds **3** and **4**[b]	–	100*	70*	8
		(116)	(152)	(132)

Deterrence rate (%)=$(C-T)/(C+T)$, where C and T are the numbers of ants feeding on the control and test solution, respectively. Absolute values of $C+T$ are given between brackets.

[a] Saponins isolated from *H. viridis*

[b] Saponins isolated from *H. foetidus*

[c] Data from Boevé and Schaffner (2003)

En dashes (–) Not tested

*$P<0.05$, Wilcoxon matched-pairs signed-ranks test

1974; Tschesche et al., 1984). Because a large majority of Phymatocerini sawflies are specialists on members of Liliales, Asparagales, or Ranunculaceae, it is likely that the steroidal saponins and alkaloids played an essential role in the evolution and radiation of the Phymatocerini. Moreover, larvae and females of *Rhadinoceraea nodicornis* are able to perceive steroidal alkaloids (Barker et al., 2002; Barker and Schaffner, unpublished data), indicating that the sequestered plant secondary metabolites also play a role in host plant selection. There are, however, a few apparent exceptions to this pattern within the Phymatocerini. For example, *Rhadinoceraea micans* and *R. reitteri* feed on *Iris*, a plant genus from which no steroidal saponins or steroidal alkaloids have been described so far. This encourages us to further investigate the influence of chemoecological parameters on radiation of Phymatocerini sawflies.

Acknowledgement We thank Prof. N. de Tommasi (Dipartimento di Scienze Farmaceutiche, Università di Salerno, Italy) for advice and registering the NMR spectra available for our studies; Dr A. Taeger for advice on sawfly identification; and Prof. M. Hilker and two anonymous referees for their constructive reviews. The authors acknowledge the financial support provided by the European Community's Improving Human Potential Programme under contract HPRN-CT-1999-00054 (INCHECO) and the Swiss Bundesamt für Bildung und Wissenschaft.

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J Chem Ecol (2007) 33:525–539
DOI 10.1007/s10886-006-9236-3

Electrophysiological and Behavioral Activity of Secondary Metabolites in the Confused Flour Beetle, *Tribolium confusum*

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Received: 17 February 2006 / Revised: 31 October 2006 / Accepted: 8 November 2006 /
Published online: 31 January 2007
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Abstract Several previous studies have addressed pheromone communication in various flour beetles (Coleoptera: Tenebrionidae), including the confused flour beetle, *Tribolium confusum* (du Val). Different stereoisomers of 4,8-dimethyldecanal (DMD) were reported as the only components of an aggregation pheromone, but the behavioral activity of DMD is low. In the present study, additional previously reported secondary metabolites (benzoquinones and hydrocarbons) were tested for electrophysiological activity (EAG) with both sexes of *T. confusum*. Two benzoquinones and three monoenic hydrocarbons elicited significant EAG activity from both male and female antennae. There was an elevated male EAG response (vs. the females) to two out of the three hydrocarbons and for both quinones. The EAG-active compounds were subsequently investigated for behavioral activity in a walking bioassay. Benzoquinones are considered toxic and have been assigned a function as alarm substances in flour beetles, but we found that methyl-1, 4-benzoquinone in intermediate concentrations was attractive to both male and female beetles and could therefore act as an aggregation pheromone component. Males were also attracted to ethyl-1,4-benzoquinone. The corresponding hydroquinones, presumed precursors of the benzoquinones, did not elicit any electrophysiological response and were not tested for behavioral activity. The unsaturated hydrocarbons (1-tetradecene, 1-pentadecene, and

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1-hexadecene) elicited significant EAG responses from both male and female antennae and were also attractive in the behavioral assay. Our results show that several beetle-produced compounds, in addition to 4,8-dimethyldecanal, may be part of a complex pheromone system in flour beetles and play a role in mediating aggregation in *T. confusum*.

Keywords *Tribolium confusum* · Tenebrionidae · Electroantennography (EAG) · Behavior · Chemical communication · Benzoquinones · Hydrocarbons · Aggregation pheromone · Dimethyldecanal

Introduction

Beetles of the genus *Tribolium* (Coleoptera: Tenebrionidae) are cosmopolitan and common pests in grain stores and flour mills. Numerous studies have investigated beetle-produced volatiles and their behavioral activity in several species of this genus, including *Tribolium confusum* (e.g., Ryan and O'Ceallachain, 1976), *Tribolium castaneum* (Herbst) (e.g., Loconti and Roth, 1953), and *Tribolium destructor* (Uyttenboogast) (e.g., Howard, 1987).

Aggregation behavior is elicited by 4,8-dimethyldecanal (DMD) in several *Tribolium* species (Suzuki, 1980; Suzuki and Sugawara, 1979; Suzuki et al., 1984, 1987; Arnaud et al., 2002). Theoretically, there are four different stereoisomers of DMD, and it has been hypothesized that the isomeric composition may differ among species. Response rates of both males and females of the confused flour beetle *T. confusum* were highest to the 4*R*,8*R* isomer both electrophysiologically and behaviorally (Levinson and Mori, 1983), whereas an 8:2 mixture of 4*R*,8*R*- and 4*R*,8*S*-DMD has been reported to mimic the aggregation pheromone of *T. castaneum* (Suzuki et al., 1984). The behavioral response to DMD is, however, low and seems to leave room for additional compounds to be identified as components of an efficient pheromone communication system (Olsson et al., 2006). Furthermore, traps baited with DMD have so far had limited success for applied purposes, which underlines the need for development of new attractants for this important group of insect pests.

Several previously identified beetle-produced volatiles may be pheromone component candidates. In addition to DMD, adults of the confused flour beetle, *T. confusum* produce benzoquinones (Engelhardt et al., 1965; von Endt and Wheeler, 1971; Suzuki et al., 1988) and volatile hydrocarbons (von Endt and Wheeler, 1971; Keville and Kannowski, 1975; Suzuki et al., 1975; Howard, 1987) (Table 1). The behavioral activity of benzoquinones has been examined for both *T. castaneum* and *T. confusum* (Loconti and Roth, 1953; Mondal and Port, 1984; Faustini and Burkholder, 1987). Most studies agree that these are defensive compounds with a repellent effect on conspecific larvae and adults (Mondal, 1985). They have also been suggested to counteract the aggregation effect of DMD (Faustini and Burkholder, 1987). The most studied unsaturated hydrocarbon produced by *Tribolium* flour beetles, 1-pentadecene, has been examined in the context of cuticular lipids and defensive secretions of flour beetles (Keville and Kannowski, 1975; Tschinkel, 1975; Suzuki et al., 1975, 1988; Lockey, 1978; Markarian et al., 1978). Different functions of 1-pentadecene have been proposed, e.g., bio-solvent for benzoquinones facilitating their absorption in the cuticle (von Endt and Wheeler, 1971; Markarian et al., 1978), repellent (Suzuki et al., 1975), or copulation pheromone (Keville and Kannowski, 1975).

We hypothesized that the *T. confusum* aggregation pheromone is more complex than what has been reported and that the previously identified quinones and unsaturated

Table 1 Previously reported secondary metabolites produced by *Tribolium confusum* and relative amounts emitted

Compound	Abbreviation	Relative Content (in %) per Class of Chemicals
Benzoquinones		
Ethyl-1,4-benzoquinone	EBQ	72.0[a]
Methyl-1,4-benzoquinone	MBQ	28.0[a]
Unsaturated hydrocarbons		
1-Tetradecene	1-C14:1	Traces[b]
1-Pentadecene	1-C15:1	89.7[b]–95.2[c]
1,6-Pentadecadiene	1,6Z-C15:2	Traces[b]-1.9[c]
1-Hexadecene	1-C16:1	0.4-0.5[b,c]
1,7-Hexadecadiene	1,7Z-16:2	<0.01[c]
1-Heptadecene	1-17:1	0.8[c]–9.7[b]
1,8-Heptadecadiene	1,8Z-C17:2	0.2[b]-1.3[c]
1,8,11-Heptadecatriene	1,8Z,11Z-C17:3	Traces[b]-0.3[c]

[a] Pappas and Morrison (1995).

[b] Suzuki et al. (1975).

[c] Howard (1987).

hydrocarbons are potential attractive pheromone components. The activity of these compounds to male and female beetles was examined by using electroantennography (EAG) and an open-arena walking bioassay and compared to the activity of DMD under the same conditions.

Methods and Materials

Insects T. confusum beetles were reared in 2.5 dm^3 jars half-filled with a 4:1 (wt:wt) mix of whole-wheat flour and Brewer's yeast, in a climate chamber at 23°C, 60% RH, and with a photoperiod of L17:D7. They were separated into males and females as adults (Ho, 1969) at least 2 wk before the bioassays were performed and were between 6 mo and 1 yr old when tested.

Chemicals Two stereoisomers of 4,8-dimethyldecanal, the (4*R*,8*R*)- and the (4*R*,8*S*)-isomers [(4*R*,8*R*)-DMD and (4*R*,8*S*)-DMD, respectively, Fig. 1] were prepared (Suzuki, 1981; Suzuki et al., 1983, Fig. 1). Thus, (4*R*,8*R*)-DMD was obtained starting from the (*R*)-enantiomer of 2-methylbutanol [(*R*)-**1**] and commercially available (*S*)-citronellal [(*S*)-**2**]. The alcohol (*R*)-**1** (not commercially available) was obtained by using a lipase-catalyzed resolution (Nordin et al., 2000) of readily available racemic 2-methylbutanol (*rac*-**1**, Fluka, Sigma-Aldrich Sweden AB, Stockholm, Sweden). A twofold molar excess of vinyl acetate and *rac*-**1** (100 g) were allowed to react in pentane (1 L), catalyzed (E=2.56) by powdered lipase PS "Amano" [10 g (Amano Enzyme Inc., Nagoya, Japan)]. The reaction was stirred at room temperature until 92% conversion was reached. The enzyme powder was filtered off, and the clear filtrate was poured onto a chromatography column (Silica gel 60, 230–400 mesh, Fluka, Sigma-Aldrich Sweden AB, Stockholm, Sweden). After elution with an ether/pentane gradient (1 to >5% ether), the remaining ester reagent and the produced ester (vinyl acetate and 2-methyl-1-butyl acetate, respectively) were collected. Gradient elution using ether (5 to 75%) in pentane gave the remaining unreacted (*R*)-2-methylbutanol. Re-

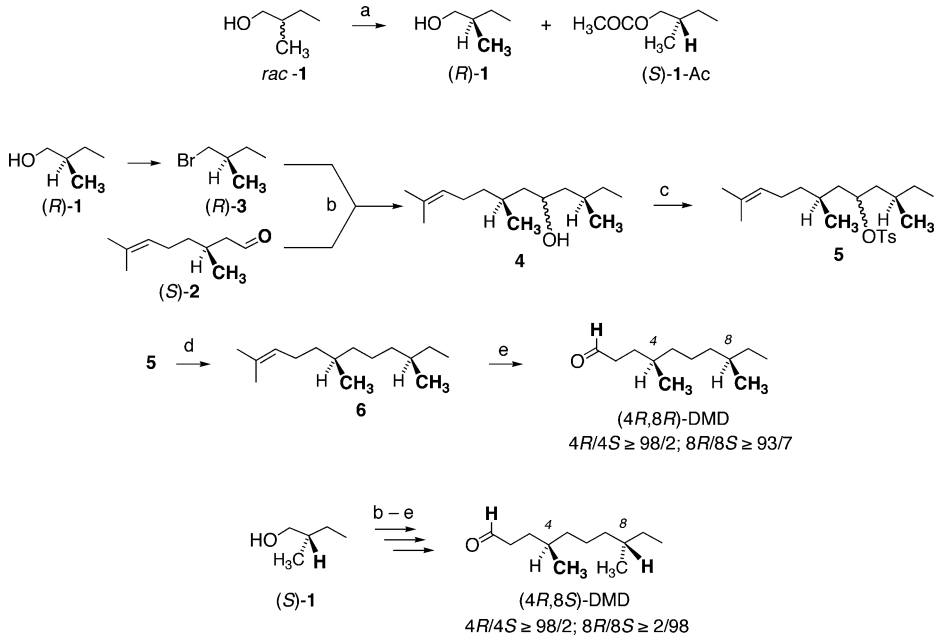


Fig. 1 Synthesis of various isomers of 4,8-dimethyldecanal. Reaction conditions: (a) Vinyl acetate, Amano PS lipase, pentane, room temperature, 2–4 d. (b)*1* (*R*)-1-Bromo-2-methylbutane [(R)-**3**], Mg, diethylether; *2* Add (*S*)-**2** in diethylether; *3* H_3O^+/H_2O, 60%. (c) TsCl/pyridine. (d) $LiAlH_4$, diethylether, 40% over two steps. (e) MCPBA, CH_2Cl_2, 0°C. (e) Dissolve in diethylether. Add $HIO_4 \cdot 2H_2O$, in tetrahydrofuran, 30% over two steps

chromatography of the alcohol fraction furnished (*R*)-**1**, 7 g [86% ee by GC, β−DEX™ 325 (Supelco, Sigma-Aldrich Sweden AB, Stockholm, Sweden), 30 m×0.25 mm i.d., d_f = 0.25 μm; carrier gas He 15 psi, split ratio 30:1, 40°C/20 min, 0.5°C/min, 50°C]. Retention time (min): 33.5 (*R*)-**1** and 34.9 (*S*)-**1**. The resolution experiment was repeated, but the reaction was interrupted at 72% conversion to give a sample of (*R*)-**1** of lower enantiomeric purity (20 g, 60% ee). The alcohol (*R*)-**1** was converted to the corresponding bromide (*R*)-**3**, which was transformed into the Grignard reagent, and reacted (Suzuki, 1981; Suzuki et al., 1983) with commercially available (*S*)-citronellal, [(*S*)-**2**] (Sigma-Aldrich Sweden AB, Stockholm, Sweden), approximately 97.5% ee by GC, [β-DEX™ 225 [(Supelco, Sigma-Aldrich Sweden AB, Stockholm, Sweden), 30 m×0.25 mm i.d., d_f=0.25 μm; carrier gas He 15 psi, split ratio 30:1, 90°C/20 min, 1°C/min, 120°C. Retention time (min): 37.4 (*S*)-**2** and 37.9 (*R*)-**2**]. This reaction sequence furnished the alcohol **4** as a mixture of diastereomers, which was converted to the tosylate **5**. This was reduced with lithium aluminum hydride to furnish the alkene **6**, which was subjected to epoxidation followed by periodate mediated oxidative cleavage to yield (4*R*,8*R*)-DMD. Thus, (*R*)-**1** (86% ee) gave (4*R*,8*R*)-DMD as a stereoisomeric mixture (>99% chemical purity by GC) with a calculated (4*R*,8*R*)/(4*R*,8*S*)/(4*S*,8*R*)/(4*S*,8*S*) ratio of 91/7/2/0 (based on the stereoisomeric purities of the starting materials), below called 4*R*,8*R*-DMD. Similarly, (*R*)-**1** (60% ee) furnished (4*R*,8*R*)-DMD as a mixture with a (4*R*,8*R*)/(4*R*,8*S*)/(4*S*,8*R*)/(4*S*,8*S*) ratio of 78/20/2/0.3 (>98% chemical purity by GC), below called an 8:2 mixture of 4*R*,8*R*-DMD/4*R*,8*S*-DMD. We prepared the other naturally occurring stereoisomer of tribolure [(4*R*,8*S*)-DMD] from (*S*)-**2** and (*S*)-2-methylbutanol [(*S*)-**1**] (both compounds from Sigma-Aldrich Sweden AB,

Stockholm, Sweden, and both approximately 96% ee by GC), by using the same reaction sequence, but excluding the first reaction, the resolution step (step a, Fig. 1). This resulted in a mixture of stereoisomers (>97% chemical purity by GC) with an approximate (4*R*,8*R*)/(4*R*,8*S*)/(4*S*,8*R*)/(4*S*,8*S*) ratio of 2/96/0/2 (based on the stereoisomeric purities of the starting materials), below called 4*R*,8*S*-DMD. The physical properties of our samples were identical to those described earlier (Mori et al., 1983, 1985).

Methyl-1,4-benzoquinone (MBQ) and methylhydroquinone (MHQ) were commercially available (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and were used as received. Ethylhydroquinone, EHQ, and ethyl-1,4-benzoquinone, EBQ, were obtained (Fig. 2) from 2,5-dihydroxyacetophenone, **7** (Sigma-Aldrich Sweden, AB, Stockholm, Sweden). Thus, when subjected to Clemmensen reduction, compound **7** gave ethylhydroquinone, EHQ. After recrystallization from toluene, colorless crystals were obtained (approximately 99% chemical purity by GC). M.p. 104–6°C (Alberola et al., 1980: m.p. 110°C). EHQ was oxidized by chromium trioxide in acetic acid/water to give crude ethyl-1,4-benzoquinone, EBQ, as a deep orange oily crystal mass. Sublimation to a cold finger in vacuo (approximately 100 mmHg) yielded bright yellow needles (>99% purity). M.p. 34°C (Harman and Cason, 1952: m.p. 37°C).

Four synthetic monounsaturated hydrocarbons were obtained commercially: 1-Tetradecene (C14:1) (Sigma-Aldrich Germany GmbH, Germany), 1-pentadecene (C15:1) (Acros Organics, Geel, Belgium), 1-hexadecene (C16:1) (Fluka Chemika, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), and 1-heptadecene (C17:1) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) (chemical purity >97% by GC).

The DMD samples and the hydrocarbons were dissolved in redistilled *n*-hexane, whereas quinones and hydroquinones were dissolved in methanol (Table 2). The purities and concentrations of the synthetic compounds were determined by coupled gas chromatography-mass spectrometry. The gas chromatograph, HP 5890 series II was equipped with an HP1 nonpolar cross-linked polydimethylsiloxane column [(Hewlett Packard Sverige AB, Kista, Sweden), 30 m×0.25 mm i.d.×0.25 μm film thickness] and interfaced with an HP 5792A mass spectrometer working in scan mode. Helium was used as carrier gas. The initial temperature was maintained at 50°C for 2 min and then increased by 10°C/min to the final temperature of 250°C.

Electrophysiology (EAG) The *T. confusum* antenna was carefully excised from the head, so that all the segments and the basal nerve were still attached to the antenna. The antenna was mounted between two glass capillary electrodes (1.5 mm o.d., 1.2 mm i.d.) filled with Ringer solution, in contact with a silver wire. The base of the antenna was first inserted into the ground glass electrode. The tip of the recording electrode was bowl-shaped, and half of the last distal antennal segment was immersed into the saline solution. The DC potential was recorded on a computer using a custom-built amplifier (Hanson B-102), and an IDAC A/D converter and software (Auto Spike v. 3.0) purchased from Syntech, Hilversum, The

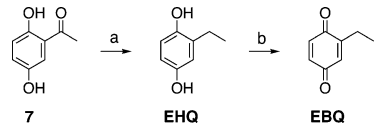


Fig. 2 Synthesis of ethylhydroquinone (EHQ) and ethyl-1,4-benzoquinone (EBQ) . Reaction conditions: (a)*1* Zn foil $HgCl_2$ HCl (aq., 1 M), decant; *2* Add HCl (aq., 5 M) and compound **7**, reflux 8 hr. (b) Cpd EHQ in HOAc, add CrO_3 in HOAc/H_2O (1/1), sublimation

Table 2 Quantities of benzoquinones and unsaturated hydrocarbons used with *Tribolium confusum*

Compound	Tested Doses	
	EAG (ng)	Behavioral Assay (ng)
Benzoquinones		
Methyl-1,4-benzoquinone	10^0–10^6	10^0–10^4
Methylhydroquinone	10^0–10^6	Not tested
Ethyl-1,4-benzoquinone	10^0–10^5	10^{-1}–10^4
Ethylhydroquinone	10^0–10^5	Not tested
Unsaturated hydrocarbons	10^1–10^7	10^{-1}–10^4

Netherlands. Filtered and humidified air was continuously flowing over the mounted antenna (approximately 0.5 m/s). A Pasteur pipette, containing a 0.5-cm^2 piece of filter paper impregnated with a 10-µl solution of the test chemical in a defined amount, was used to deliver the stimulus in a 2.5-ml puff during 0.5 s by means of a stimulus controller (CS-02, Syntech, Hilversum, The Netherlands). The solvents used for the stimuli were tested alone and elicited minimal EAG responses, indistinguishable from responses to air. Ten micrograms of two DMD isomers, 4*R*,8*R*- and 4*R*,8*S*-DMD, and also an 8:2 mixture of 4*R*,8*R*-DMD to 4*R*,8*S*-DMD were tested initially. The EAG responses were recorded every 3 min after the excision to test the longevity of the antennal preparation. This experiment was followed by an EAG study of the response to different doses of the most active isomer.

In the following experiments, an active control consisting of 10 µg of 4*R*,8*R*-DMD was applied before each test stimulus. Five seconds separated the DMD control puff and the tested substance puff, and 10 sec separated two tested doses. Every EAG response will, therefore, be presented as a normalized response that corresponds to the ratio between the EAG response induced by the tested solution of benzoquinones/hydrocarbons and the response induced by 10 µg 4*R*,8*R*-DMD. Each experiment was conducted in five replicates for both sexes.

Behavioral Assay The open-arena olfactometer was a modified version of a walking bioassay previously used for investigations of beetle behavior (Lanne et al., 1987; Olsson et al., 2006). The olfactometer was 0.8 m long and 0.2 m wide. A source of compressed air provided a laminar airflow of 0.2 m/s over the arena and an air exhaust evacuated contaminated air to the outside of the building. The floor of the arena was made of a glass plate on which a sheet of paper was placed. This paper was replaced between tests of different compounds. The test compound was released from a 1-µl glass capillary (32 mm long, 0.1 mm i.d.) (Drummond, MICROCAPS®, Drummond Scientific Co., Broomall, PA, USA), which was suspended over, but not touching, the paper at the upwind end of the arena. Two capillaries were used when testing the benzoquinones. Whereas all the methanol solvent had evaporated from the capillary within 10 min, evaporation of the *n*-hexane required only 5 min. Actual release rates of the test compounds are not known. The laboratory temperature and relative humidity were controlled at 22.5±0.5°C and 60±5%, respectively.

Ten beetles of the same sex were put in a vial. Ten vials, five for each sex, were used to test one dose of a chemical. The vial was placed in the center of the olfactometer and was left for 30 sec for beetles to acclimate. The capillary was filled with the solution and beetles were released and allowed to respond during 5 min. At the end of the test, beetles collected upwind from the release point were counted as responding/attracted (beetles approaching the capillary within the 5 min were withdrawn before they could touch the odor source and

then counted as responding). New beetles were used in each test, so that one beetle was not tested more than once a day. The measured response in each test was calculated as the number of beetles attracted out of the ten tested. Solvent was used as a control (methanol for benzoquinones, *n*-hexane for the other compounds). An active control consisting of 1 μg of 4*R*,8*R*-DMD was tested each day to check that the beetles were behaving similarly each experimental day.

Statistical Analyses Statistical tests were performed with the statistical software Minitab® 14.0 for Windows. Analyses of variance (ANOVA) were carried out for both the electrophysiological and behavioral experiments. Three factors were tested initially (antenna, sex, and dose of tested chemical) for the electrophysiological trials, whereas two factors (sex and dose of tested chemical) were tested in the behavioral trials. General linear model (GLM) (Wolfinger and Chang, 1995) was used instead of ANOVA when unbalanced datasets were analyzed. Differences between means were tested for significance with Dunnett's (comparison with a control) or Newman and Keuls' (comparison by groups of means) tests.

Results

DMD, Choice of the Active Control for Subsequent Experiments Antennal activity decreased significantly with time after the excision of the antenna in both sexes (females, $F_{4,60}$=167.14, P<0.001; males, $F_{4,60}$=208.49, P<0.001) (Fig. 3). There were differences in both female ($F_{2,60}$=2.48, P=0.022) and male ($F_{2,60}$=43.65, P<0.001) EAG activities among the three stimuli. Due to their EAG activities, the two isomers and the blend were tested behaviorally (Fig. 4). There was no difference in walking behavior between the

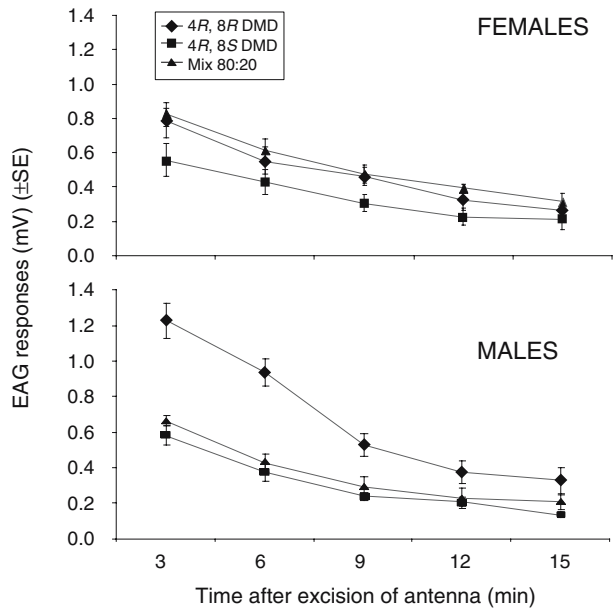


Fig. 3 Effect of time on mean EAG responses (±SE) of female and male *Tribolium confusum* antennae (N=5) to 10 μg of 4*R*,8*R*-DMD, 4*R*,8*S*-DMD, or an 80/20 blend of the two isomers

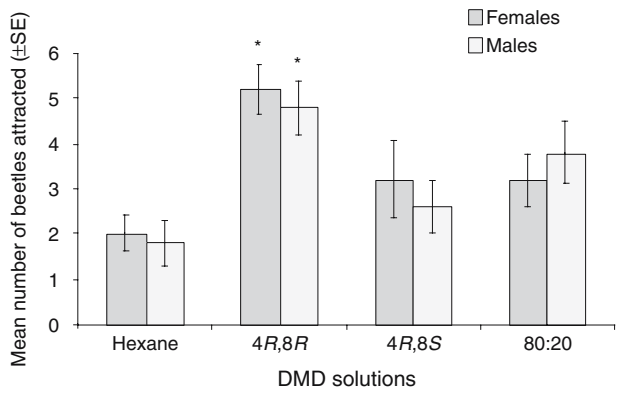


Fig. 4 Mean behavioral responses (±SE) of *Tribolium confusum* (N=5) to 1 μg of 4*R*,8*R*-DMD, 4*R*,8*S*-DMD, or an 80/20 blend of the two isomers. *n*-Hexane was used as control. * indicates significant difference from the control within each sex (Dunnett's test, P<0.001)

sexes, but the responses to the two isomers and the blend were different ($F_{4,40}$=7.42, P< 0.001), and 4*R*,8*R*-DMD was more attractive than the *n*-hexane control (Dunnet's test, P<0.001).

EAD and Behavioral Dose–response to 4R,8R-DMD 4*R*,8*R*-DMD was the most active and attractive isomer, so it was tested in different concentrations in both EAG and behavioral assays. There was a positive dose–response relationship in EAG for both females ($F_{5,80}$= 105.8, P<0.001) and males ($F_{5,80}$=30.7, P<0.001). The two highest doses (1 and 10 μg) elicited higher EAG responses than the *n*-hexane control in both females (Dunnet's test, P< 0.001) and males (Dunnet's test, P<0.001) (Fig. 5). The highest dose (1 μg) induced a higher walking response in both females and males (Dunnet's test, P<0.001), whereas 0.1 μg induced a higher response than the control (Dunnet's test, P<0.05) for females only (Fig. 6). There were no differences between male and female responses.

Benzoquinones Both MBQ and EBQ elicited higher EAG responses than the control (Fig. 7) ($F_{6,52}$=20.97, P<0.001 and $F_{5,44}$=13.25, P<0.001, respectively). There were also significant differences in EAG responses between the sexes for both MBQ ($F_{1,52}$=4.58, P= 0.037) and EBQ ($F_{1,44}$=10.25, P=0.003); males were more sensitive. In the walking assay, MBQ attracted both sexes (GLM, $F_{5,68}$=8.42, P<0.001) compared to methanol (Fig. 8).

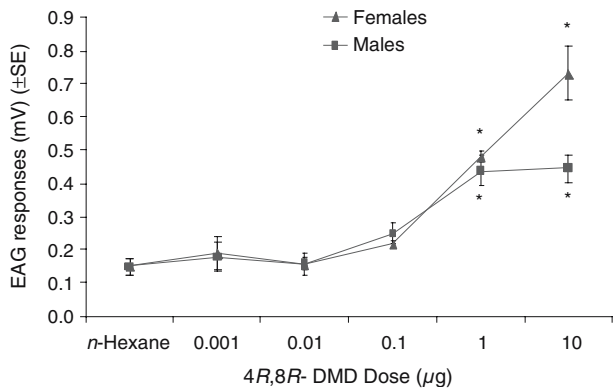


Fig. 5 Effect of 4*R*,8*R*-DMD dose on the antennal responses (±SE) of female and male *Tribolium confusum* (N=5). * indicates significant difference from the *n*-hexane control within each sex (Dunnett's test, P<0.001)

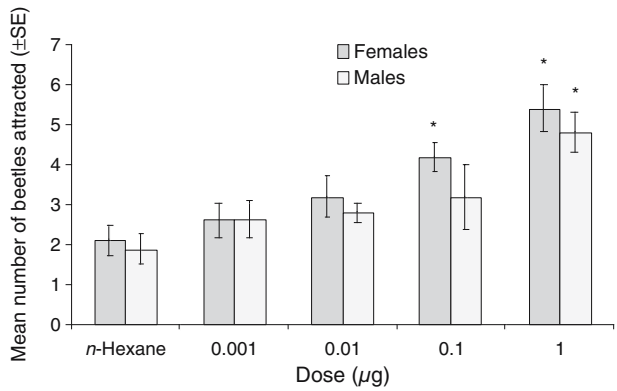


Fig. 6 Mean behavioral responses (±SE) of *Tribolium confusum* (N=5) to increasing doses of 4*R*,8*R*-DMD. *n*-Hexane was used as control. * indicates significant difference from the *n*-hexane control within each sex (Dunnett's test, P<0.05)

There was no difference in behavior between males and females in response to MBQ ($F_{1,68}$=1.61, P=0.209), whereas there was an interaction between dose and sex with EBQ ($F_{6,76}$=4.69, P<0.001). Female and male attraction were found to be dose-dependent for EBQ ($F_{6,38}$=4.05, P=0.003 and $F_{6,38}$=6.03, P<0.001, respectively). The EAG experi-

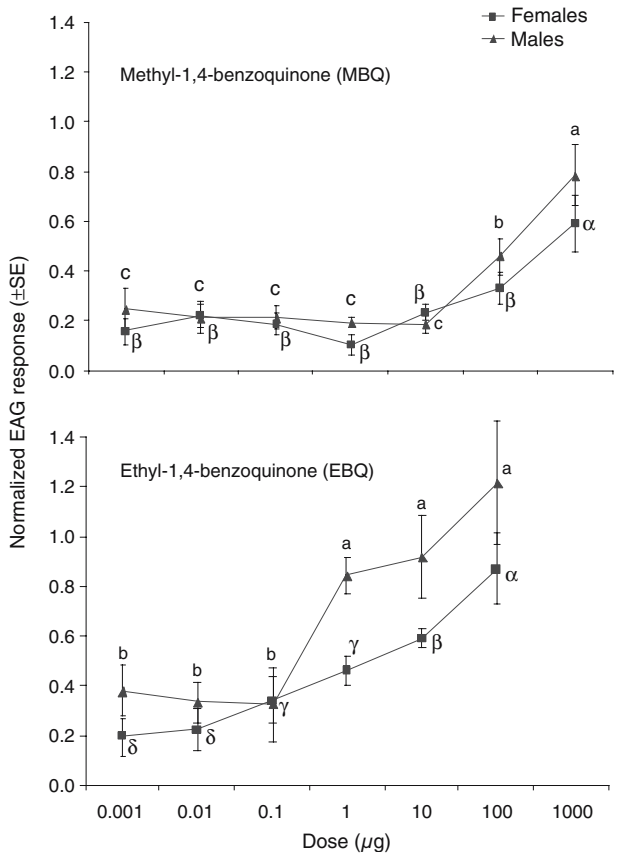


Fig. 7 Effect of doses of methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone on antennal responses (±SE) of female and male *Tribolium confusum* (N=5). EAG responses to benzoquinones have been normalized to the 10 μg 4*R*,8*R*-DMD responses. Means with the same letter within each sex are not significantly different (Newman and Keuls test; α=0.05, N=5, df=24)

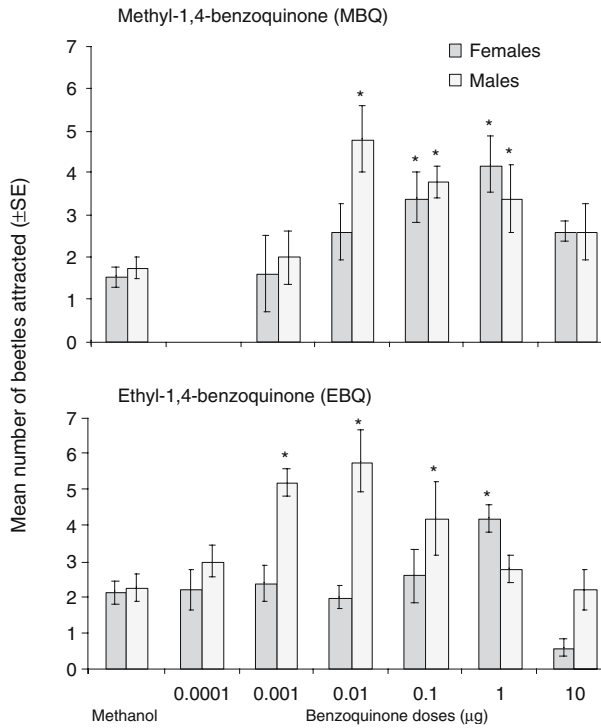


Fig. 8 Mean behavioral responses (±SE) of *Tribolium confusum* (N= 5) to increasing doses of methyl-1,4-benzoquinone and ethyl-1,4-benzoquinone. Methanol (1 µl) was used as control. * indicates means that are significantly different from the methanol control (Dunnett's test, P<0.05)

ment with the benzoquinone precursors, the hydroquinones, revealed no activity for any of the doses of MHQ or EHQ. These substances were, therefore, not tested behaviorally.

1-Tetradecene Female and male antennal responses were dependent on the dose of C14:1 ($F_{6,24}$=13.99, P<0.001 and $F_{6,24}$=21.93, P<0.001, respectively) (Fig. 9). The male response was higher than the female response at the higher doses. In the behavioral assay, the beetles displayed a positive dose–response to C14:1 ($F_{6,76}$=7.84, P<0.001), and there was no difference between the sexes (Fig. 10).

1-Pentadecene Female and male antennae responded differently, generally with higher male responses, to different doses of C15:1 in the EAG experiment ($F_{6,24}$=56.03, P<0.001 and $F_{6,24}$=16.86, P<0.001, respectively) (Fig. 9). In the walking assay, both sexes displayed a dose-dependent attraction to C15:1 ($F_{6,76}$=5.02, P<0.001), but no difference between the sexes was observed (Fig. 10).

1-Hexadecene Males and females responded similarly in the EAG experiment to C16:1 (Fig. 9). The dose had an effect on the antennal responses of both sexes ($F_{6,52}$=10.93, P< 0.001). The longer homolog C17:1 elicited no significant EAG response (data not shown). Both males and females were attracted to C16:1 in the walking bioassay (GLM, $F_{6,76}$=4.29, P<0.001) (Fig. 10).

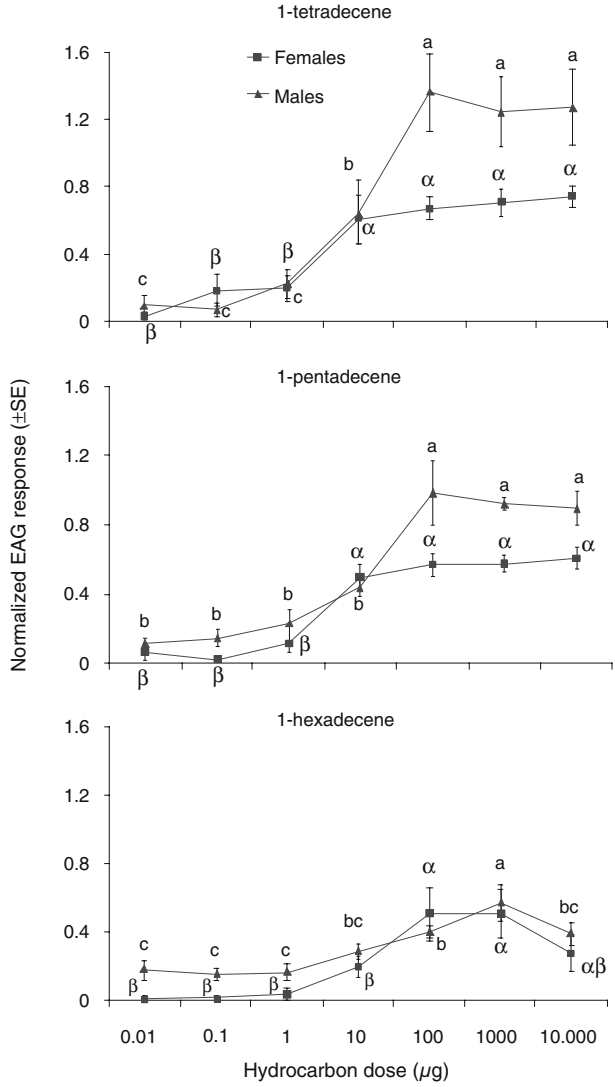


Fig. 9 Effect of doses of 1-tetradecene, 1-pentadecene, or 1-hexadecene on antennal responses (±SE) of female and male *Tribolium confusum* (N=5). EAG responses to hydrocarbons have been normalized to the 10 μg 4*R*,8*R*-DMD responses. Means with the same letter within each sex are not significantly different (Newman and Keuls test; α=0.05, N=5, df=24)

Discussion

This study of secondary metabolites from the confused flour beetle *T. confusum* shows that several semiochemicals, in addition to the previously identified aggregation pheromone DMD, elicit antennal and behavioral responses in both sexes and may comprise a more complex pheromone system. Both electrophysiological and behavioral responses of male and female *T. confusum* were highest to the 4*R*,8*R* isomer of DMD, in agreement with the work of Levinson and Mori (1983). However, benzoquinones, commonly referred to as defensive secretions or inhibitors of microbial growth in the flour beetles (Engelhardt et al., 1965; Faustini and Burkholder, 1987; Mondal, 1990), were also found to be EAG-active

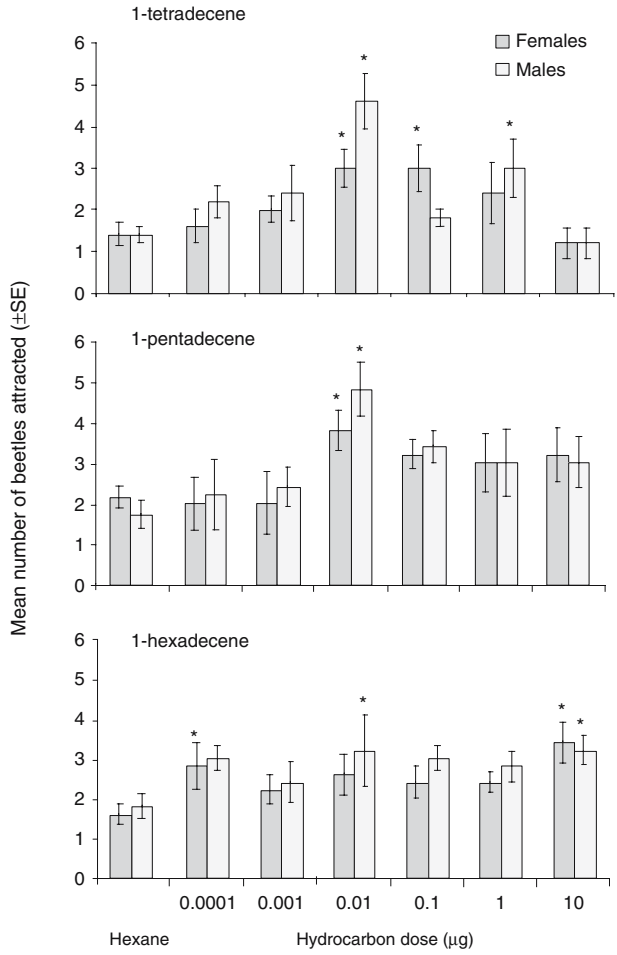


Fig. 10 Mean behavioral responses (±SE) of *Tribolium confusum* (N=5) to increasing doses of 1-tetradecene, 1-pentadecene, or 1-hexadecene. *n*-Hexane (1 µl) was used as a control. * indicates means significantly different from the *n*-hexane control (Dunnett's test, $P<0.05$)

and as attractive as 4*R*,8*R* DMD, especially to males. Furthermore, two unsaturated hydrocarbons elicited antennal responses and attraction of both female and male beetles.

A dense population of flour beetles could potentially produce substantial amounts of the benzoquinones. Engelhardt et al. (1965) estimated that when subjected to stress, e.g., extreme temperature, the amounts of these compounds released by *T. confusum* were around 1 µg/beetle/3 hr. Our results suggest that lower concentrations attract both males and females, whereas higher concentrations reduce attraction. The reduced attraction to the high concentrations is in agreement with Faustini and Burkholder (1987), who reported that higher concentrations can cause the beetles to disperse. It seems adaptive that the beetles have evolved different behavioral responses to different concentrations of benzoquinones, and these compounds may serve as a spacing pheromone at higher concentrations, similar to what has been reported for other compounds in aphid chemical ecology (e.g., Quiroz et al., 1997; Gonzales et al., 1999).

Antennal receptors in *T. confusum* responded to the three unsaturated hydrocarbons (C14:1, C15:1, C16:1). Male antennae had stronger responses to the highest doses of the

C14:1 and C15:1 than female antennae. Both EAG and behavioral responses decreased with increasing chain length, and the attraction to C16:1 is low in both sexes, which could partly be explained by the corresponding decrease in volatility of the stimuli. The attraction of males (and females) to the hydrocarbons agrees with the male response to pentadecene and heptadecene reported by Keville and Kannowski (1975). They found that males mounted other males and females upon stimulation with these volatiles. In contrast, they did not find any female response to the hydrocarbons, whereas in our study, females responded both electrophysiologically and behaviorally. Suzuki et al. (1975) reported that the hydrocarbons in question were repulsive when 10 μg were deposited on a filter paper. Our results do not explicitly support this claim, but the maximal dose used in our experiments (10 μg) did, at least, not elicit any significant attraction. Comparisons between tests using different dispensers are difficult to make as application of similar doses does not necessarily imply the same actual emission. We conclude that, similarly to the benzoquinones, the three hydrocarbons (C14:1, C15:1, and C16:1) were as attractive as the previously reported aggregation pheromone DMD in the behavioral assay. Unsaturated hydrocarbons could thus play an aggregative role when released at low doses complementary to the benzoquinones.

Gas chromatography in combination with electroantennographic detection (GC-EAD) should have been a suitable method to test the relative activity of precisely quantified amounts of various pheromone candidates and also to examine insect extracts for potential pheromone component candidates. However, we could not use GC-EAD for measuring the activity of different compounds at different concentrations as the lifetime of antennal preparations was short and variable. Regular use of this approach would have allowed the introduction of a better-defined amount of the stimulus, but would at the same time have introduced new problems when it came to the recording of the actual response. GC-EAD analyses of natural samples were also not productive. GC-EAD recordings from *T. confusum* will require major improvements of the antennal preparation.

The likely mixed function of semiochemicals produced by flour beetles is similar to what has been reported for secretions of sternal glands in caddisflies (Trichoptera). Several compounds produced in these glands show high fumigant activity and have been assigned a function in chemical defense (Ansteeg and Dettner, 1991). Compounds produced in the same glands, and in some cases, even the same compounds as those reported by Ansteeg and Dettner (1991) have been confirmed as components of sex and aggregation pheromones, respectively, in *Rhyacophila fasciata* and *Hydropsyche angustipennis* (Löfstedt et al., 1994).

The chemical communication of the *Tribolium* flour beetles appears complex, and several classes of chemicals are involved in their behavior. The present study shows that DMD is not the only compound produced and attractive for *T. confusum*. This raises the question of the natural composition of the aggregation pheromone of these beetles. Indeed, *T. confusum* aggregation pheromone seems to be composed of a blend of chemicals, which depending on emitted amounts, are able to attract the beetles as efficiently as DMD. Further studies should focus on experiments involving several of the active chemicals to highlight increased attraction or even possible synergy.

Acknowledgments The authors thank the Swedish Foundation for Strategic Environmental Research (MISTRA), the Cerealia R&D Foundation and Erasmus for funding this project. We thank Amano Enzyme Inc., Japan, for a generous gift of lipases. We are also grateful to Dr. Y. Brostaux (Department of Statistic and Computer Sciences, Gembloux Agricultural University) for his advice on statistical analysis. Ludovic Arnaud was funded by the FNRS (Fonds National de la Recherche Scientifique), OECD (Organisation for Economic Cooperation and Development).

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J Chem Ecol (2007) 33:541–554
DOI 10.1007/s10886-006-9243-4

Position Around A Tree: Consequences For Pheromone Detection

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Received: 9 June 2006 / Accepted: 18 December 2006 /
Published online: 25 January 2007
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Abstract The air flow pattern expected around a cylindrical object such as a tree in slow wind, is predicted from fluid mechanics to have areas of faster flow (upwind) and slower recirculating flow with eddies (downwind). An organism located on the surface of a tree would experience different flow depending on its circumferential position. If that organism was searching for a chemical signal, such as a pheromone plume, it might maximize its probability of chemodetection by placing itself in areas of greatest flow speed (the upwind surface of the cylinder, i.e., in front of the separation points). We tested whether wood cockroaches in the genus *Parcoblatta* exhibit such upwind positioning; they live in forests, and males actively fly from tree to tree, while searching for females releasing sex pheromone. In contrast to an expectation of upwind preference, male cockroaches were evenly distributed around trees relative to upwind (measured with a novel "feather boa" flow visualization technique), even though the wind direction was relatively steady. We investigated whether sex pheromone could be detected at any location around a cylindrical surface in a laboratory flow chamber by using *Bombyx mori* wing fanning as a bioassay. Although upwind moths arrayed on the surface detected pheromone more rapidly, pheromone detection occurred at least a third of the time at any position, which could explain the even distribution of *Parcoblatta* males around trees.

Keywords Air flow · Reynolds number · Forest insect · Velocity boundary layer · Wind variability · Pheromone · Olfaction · Cockroach · Chemoreception · Flow visualization

Introduction

Many forest insects localize potential mates by the use of volatile, airborne pheromones (Elkinton et al., 1987; Brady et al., 1989; Wyatt et al., 1993, 1997). Wind is the primary

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pathway for communication via the olfactory channel; as such, it determines where pheromones will be carried. Although the pheromone molecules and the air currents that bear them are invisible, we can visualize their movements by using smoke, bubbles, and other physical markers. These visualization techniques have been vital in developing our understanding of natural airflow patterns in forests and grasslands (David et al., 1982, 1983; Elkinton et al., 1987; Willis et al., 1994; Murlis et al., 2000). Air flow patterns dictate the shape and behavior of pheromone plumes and the challenges faced by insects tracking such complex and intermittent signals. It is well-established that pheromone released by an insect travels downwind in a meandering sinuous plume as a result of wind shifts (David et al., 1982, 1983; Murlis et al., 1992, 2000). Despite the slow (<1 m/sec) and shifting air movements in a forest (Smith et al., 1972; Oliver, 1975), insects are able to track pheromone or host-odor plumes and locate odor-emitting individuals (Brady et al., 1989, 1990; Willis et al., 1994).

An animal located on a tree or other obstacle, around which pheromone-laden air is swirling, may have opportunities to intercept or miss a pheromone plume, depending on its location around the tree. Downwind of a cylindrical obstacle, the air is moving especially slowly and recirculating in eddies (Fig. 1) (Brady et al., 1989; Vogel, 1994). It follows from this flow pattern that for individuals on a surface: (1) upwind individuals should intercept pheromone first, whereas individuals further downwind on the same tree would receive a delayed and possibly diluted chemical sample; (2) as a result of these velocity differences between upwind and downwind areas, individuals can potentially change the amount of air they sample by changing their position around the cylinder. Therefore, insects on the upwind surface would be sampling a larger volume of air per unit time, which would allow an individual to increase its likelihood of encountering pheromone molecules.

To evaluate whether potential advantages of upwind positioning are utilized by a forest insect that uses volatile sex pheromones to locate a mate, we measured circumferential positions of native wood cockroaches in the genus *Parcoblatta* in a forest. These cockroaches actively court in early summer during the evenings (Hebard, 1931; Helfer, 1953; Lawson, 1967; Horn and Hanula, 2002). The short-winged (nonflying) females are found primarily on the ground and release sex pheromone (Gorton, 1980; Gemeno et al., 2003). Males are long-winged and fly from tree to tree, presumably while searching for a pheromone-releasing female (Gorton, 1981; Miller, personal observation).

In the laboratory, complementary investigations were performed to evaluate how the timing and probability of interception of pheromone plumes varied with location around a

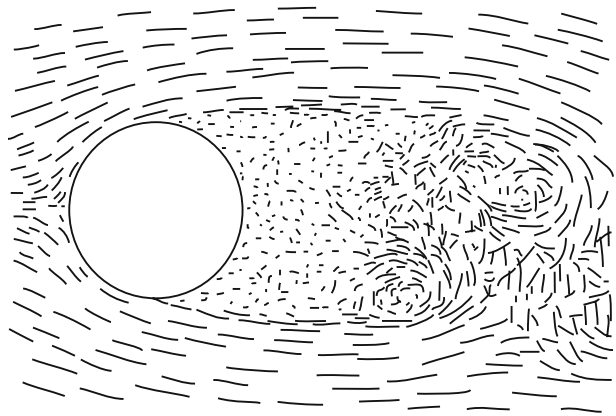


Fig. 1 The pattern of flow around a cylinder. This is visualized by using *pathlines*; a *pathline* shows the path followed by a parcel of air during a set time interval (Vogel, 1994). Flow approaches the cylinder from the left, and *longer pathlines* indicate faster flow. Flow near the downwind surface of the cylinder is much slower and contains swirling eddies. The cylinder and flow are viewed in cross-section. (*Pathlines* were drawn based on a photograph in Van Dyke, 1982, p. 31, of flow at Re=2000)

cylinder. For these measurements, we used a wing fanning assay to visualize pheromone interception by moths arrayed around a cylinder in controlled flows. Adult male *Bombyx mori* show a clear visual response (wing fanning) to detection of the sex pheromone released by the females (Loudon and Koehl, 2000).

If wind direction is shifting substantially, an organism on a tree surface would need to relocate to stay upwind. To measure the rate at which the upwind direction on a tree changed over a biologically relevant time scale (on the order of minutes or hours), we made multiple measurements of upwind direction both in a natural forest and on solitary trees in a field.

Methods and Materials

Distribution of Parcoblatta Relative to Wind Direction Orientation behavior of males from two species of *Parcoblatta* cockroaches (*Parcoblatta pennsylvanica* and *Parcoblatta uhleriana*) was observed during their mating season in June of two successive years. [Note that authorities differ on the spelling for *P. pennsylvanica*; Hebard (1917) uses *P. pensylvanica*.] The field site was a deciduous forest within the Rockefeller Experimental Tract, Lawrence, KS (Kansas Biological Survey). Male cockroaches became active (were flying around) as darkness fell. After landing on tree trunks, males often touched the tree surface briefly with their antennae before taking off or staying in place, but did not often walk far on the tree surface. Activity of these flying males declined about 4 hr after dark (males were sometimes observed to move under bark). After sighting a male on a tree surface, its circumferential position was measured with a compass, and its height above the ground was measured to the nearest centimeter (cm) with a meter stick. Upwind direction was recorded at each tree at the time the cockroach was sighted by using a novel marabou "feather boa" visualization method (marabou feathers are easily displaced, being fine and light). The boa (marabou feather boa, Hobby Lobby) was wrapped around the tree at the time and height at which the male was located, generating a circumferential wind indicator. The down-like feathers splayed apart in response to the wind, indicating upwind direction at the tree surface, which was recorded with a compass. In addition to upwind direction, the boa visualization method enabled us to determine whether the air speed was below the slow threshold air speed, indicating extremely still air. Thus, this boa method allowed measurement of wind direction, but only a qualitative (or threshold) estimate of wind speed. This method differs from the method of Baker and Cardé (1979) that used the calibrated displacement of a single feather to estimate air speed at a single location.

The response threshold of the marabou boa (minimum air speed required for a detectable response) was determined by placing it around a cylinder (9-cm diameter) placed inside a large slow-flow chamber (cross-sectional area=1,089 cm^2). The air moving through the chamber passed through a flowmeter (King Instrument Company) that was used to measure the volume flow rate, from which the average cross-sectional velocity was calculated. The response threshold of the boa, denoted by noticeable parting of feathers showing wind direction, corresponded to an average ambient flow of approximately 2.5 cm/sec (imperceptible by humans).

Variability in Upwind Direction at Tree Surfaces for Solitary Trees Wind direction variability was estimated at tree surfaces during the months of June and early July. Measurements of upwind direction were taken once every minute for solitary trees. Each of the six "solitary" trees was a minimum of 7.5 m from any neighboring tree. The variability

in wind direction over time for individual trees was estimated from multiple measurements; the upwind direction was recorded to the nearest degree at the surface of individual trees by using a compass and boa (as described above) for 10 successive measurements at 1-min intervals for each tree. Three sets of 10 successive measurements were taken at each of three different times: in the morning, midday, and evening for each tree ("morning" times were between 8 and 10:40 A.M., "afternoon" times between 12 and 2:40 P.M., and "evening" times between 8 and 10:40 P.M.). The variability in wind direction was calculated as the circular standard deviation of the 10 successive minute-by-minute measurements at a tree; the circular standard deviations were averaged for the three (or two) sets for each time of day and tree. During one morning, measurements were not taken due to rain. The circumferences of these six trees ranged from 0.17 to 1.45 m.

Variability in Upwind Direction at Tree Surfaces for Forest Trees We also determined whether upwind direction was the same for multiple trees in a group, both in the forest and at its edge, to look at the effect of neighboring trees during the month of June. We selected 10 trees along the edge of the forest and 10 trees within the forest for repeated measurements of wind direction over a 10-d period, recording upwind directions once early in the evening (8–9 P.M.) and once 1/2 to 2 1/2 hr later (weather permitting). We also compared the frequency with which the wind exceeded a slow threshold (proportion of times that wind direction was detectable using the feather boa method) between "edge" and "within forest" trees.

Experimental Animals for Pheromone Detection in Laboratory Measurements *B. mori* were reared on mulberry leaves under a natural light cycle. Once caterpillars had pupated, they were sexed, placed in individual cups, and checked daily for emergence. All males used were at least 1 d old and unmated; some males were used repeatedly by random selection from the available males. Unmated females were used as a sex pheromone source.

Experimental Setup for Controlled Flow in Laboratory Measurements Tethered male moths were arrayed around a vertical cylinder (27 cm tall and 16.6 cm diameter) centered in a flow chamber (60×84×27 cm). The moths were located halfway up the cylinder, spaced 45° apart (eight males total at a time). Moths were secured to wooden supports that passed through slits in the otherwise solid cylinder, allowing them to be positioned at the cylinder surface ("0 cm") or 5 cm out from the surface. Moths were positioned with their anterior–posterior axes oriented vertically, so that their wing fanning would drive air downwards (Loudon and Koehl, 2000) rather than around the cylinder. The flow chamber was located in a laboratory hood, which generated the air flow and exhausted the pheromone-laden air to the outside. The flow chamber was either centered within the hood opening or positioned against a side to generate different types of flow: symmetrical or asymmetrical (left/right), respectively. These different flows generated pheromone plumes of different shapes (Fig. 2). Cloth mesh covered the front of the flow chamber to slow and smooth the flow. Trials were videotaped by a camera (Sony Digital Handycam DCR-PC101) mounted above the flow chamber.

The cylinder with the array of eight moths was placed in the center of the flow chamber with one male placed directly upwind. Lights were turned on, and the moths were given time to acclimate to the flow (~20 min), because males would wing fan when they were disturbed by movement or changes in lighting. Once most males had ceased fanning, the pheromone was released into the air stream as described below. Any males that were already fanning at the beginning of the trial were not included in the analysis. The response

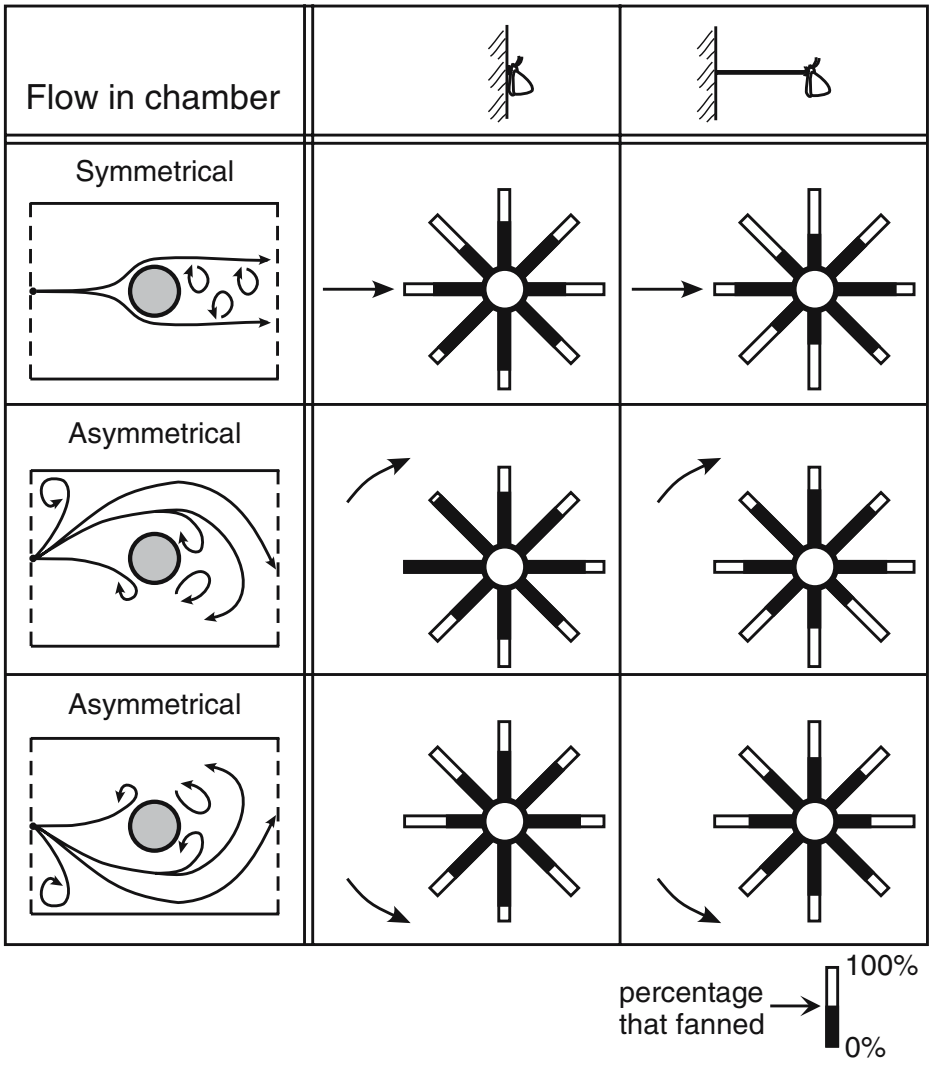


Fig. 2 Male detection of pheromone from all positions around the cylinder. On average, this detection occurred 62% of the time, as indicated by wing fanning, across all flow types and moth positions. The percentage of males that fanned is indicated by the *black fill in the bar*. A *diagrammatic representation* of the flow treatment appears in the left column as viewed from above, with the pheromone plume generated from a point source as shown. The distance of the moth from the surface of the cylinder (0 or 5 cm) is indicated in the *top row*; the moths on supports are shown as viewed from the *side*

times (onset of wing fanning) of the males were recorded for 30 sec starting with the pheromone release. The cylinder was then rotated so that a different male was now in the upwind position (order randomly assigned), the males were given at least 5 min to acclimate to new positions, and the wing fanning response times to the next pheromone plume were recorded. The cylinder was turned repeatedly until each of the eight males had been in each position once (eight rotations). These eight rotations, with eight male responses at each of the eight positions, were considered one "trial." A pool of 51 male moths was used in the 23 trials.

One to three live females visibly extruding their pheromone glands were placed into a 30-ml syringe centered at the upwind edge of the flow chamber (34 cm upwind). The amount of pheromone released by the females was unknown and presumably variable, which was not a concern because the responses at different positions around the cylinder were compared to each other, thus, providing an internal control. To generate a pheromone plume, air was passed through the syringe slowly and joined the air moving through the flow chamber. The air passed through the syringe at a volume flow rate of 9.9 ml/sec for 8 sec time interval; therefore, approximately twice the volume of the syringe was exchanged during the stimulus. The air flow through the syringe was controlled with an aquarium pump, a solenoid valve, and a custom-built timing circuit (Instrumentation Design Laboratory, University of Kansas) and was calibrated by collecting air in an underwater graduated cylinder with the same setup. A light-emitting diode (LED) light (in view of the video camera but not the moths) was illuminated for the 8 sec that air was passing through the syringe, to record the timing of the pheromone release on the videotape. For the controls (N=3 sets of eight males), air was passed through a clean syringe without a female, and the males were observed for 30 sec. The inner surface of the chamber was cleaned daily (wiped with ethanol and air-dried).

Controlled Flow in Chamber We generated slow flows within a flow chamber that contained small, ephemeral eddies to mimic those characteristics of air flow that we would expect in a natural forest (Fig. 2). We generated a symmetrical flow (Fig. 2, top) and asymmetrical flows (Fig. 2, middle and bottom) that contained a large circulating gyre in addition to the flow that separated and moved around both sides of the cylinder. These gyres were either clockwise or counterclockwise when viewed from above, depending on whether the flow chamber was placed against the right or left side of the hood opening, respectively. The large-scale gyration had the effect that the "upwind" area on the cylinder was displaced slightly to one side. Flow visualization by using smoke indicated that small-scale eddies formed and dissipated at a variety of different locations within the chamber at different times leading to a complex and slightly unpredictable flow. The temperature within the chamber ranged between 23 and 24°C.

The flow chamber was illuminated by lamps on both sides; however, lighting was nonuniform due to additional natural lighting from windows. The difference in light levels appeared to change the behavior of the males slightly, with those in brighter lighting fanning more readily. In symmetrical flow, males would be expected to fan at the same time as those opposite them. However, we found that those on the side with brighter lighting fanned earlier than males in dimmer lighting at both 0 and 5 cm from the cylinder (Wilcoxon rank sums; 0 cm, $P_{2\text{-sided}}<0.001$; 5 cm, $P_{2\text{-sided}}=0.003$; time difference was 2 sec or less). To compensate for the difference in speed of fanning as a result of differential lighting, we applied a light correction factor. Adjustments of ±0.5 or 1 sec were applied to each position (except directly upwind or downwind) and distance from the cylinder surface in all flow conditions.

Burning incense sticks were used as a smoke source to visualize air flow patterns and speeds around the cylinder and within the flow chamber. Although incense smoke is not neutrally buoyant, the horizontal component of movement is independent of its upward component. Smoke was illuminated from both sides and filmed from above. Motion analysis software (Motus 8, Vicon/Peak Performance, Englewood, CO, USA) was used to digitize smoke paths across multiple frames of video to determine how long it took smoke to travel from the syringe to the cylinder to the nearest 1/60 of a second. Note that the distance smoke traveled to reach the cylinder is not the same for symmetrical flow as it is

for asymmetrical flow, because in symmetrical flow, smoke traveled directly to the point on the cylinder closest to the syringe. Flow visualization demonstrated that there was no significant difference in the time for smoke to reach analogous points on the left vs. the right sides of the cylinder in symmetrical flow (two-way analysis of variance (ANOVA) with side and position as classes, $N=38$, $P=0.186$ for side comparison).

Flow speeds from the syringe to the cylinder estimated by smoke movements (as described above) are as follows: symmetrical 0.5 m/sec (±0.04 SD, $N=3$), asymmetrical clockwise 0.2 m/sec (±0.04 SD, $N=18$), and asymmetrical counterclockwise 0.3 m/sec (±0.6 SD, $N=18$). Therefore, it took approximately 0.5–2 sec for the pheromone to be carried from the syringe to an upwind moth, depending on the flow. For symmetrical flow, it would take approximately 2 sec for the front of a pheromone patch to leave the syringe, diverge, and be carried completely past the two sides of the cylinder. Pheromone that has passed downwind of the cylinder may be caught up in a recirculating eddy and brought back to the surface more slowly (Fig. 2). For asymmetrical flows, it would take approximately 10–20 sec for a pheromone patch to leave the syringe and swirl completely around the cylinder.

Statistical Analysis Circular statistics were performed in Oriana 2.01b (Kovach Computing Systems; Anglesey, Wales). Other statistical analyses were done by using SAS (version 8.2, Cary, NC, USA).

Results

Natural Wind Conditions and Parcoblatta Positions Male *Parcoblatta* cockroaches of both species were found in approximately equal numbers on the upwind and downwind halves of trees: *P. pennsylvanica* ($N=14$ upwind, $N=13$ downwind) and *P. uhleriana* ($N=19$ upwind and $N=24$ downwind). The distribution around the circumferences of trees with respect to the upwind direction was not different from a uniform distribution for either species (Fig. 3; Rayleigh's uniformity test for circular statistics $P>0.05$). We also tested whether *Parcoblatta* males were positioning themselves on the tree by compass direction; there was no difference from a uniform distribution for either species (Fig. 3; Rayleigh's uniformity test for circular statistics $P>0.05$). Although males from both species were found at a variety of different heights above the ground (range 0.1 to 2.3 m), the species were found at different heights on average, as has been shown earlier (same species,

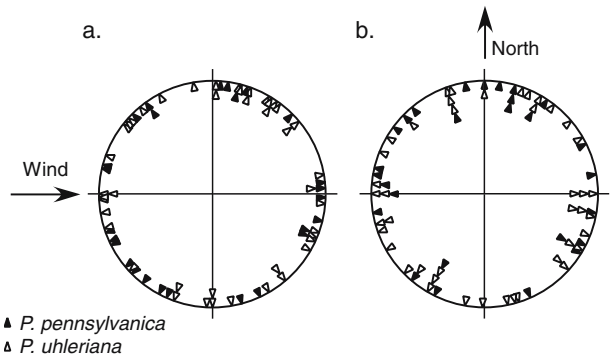


Fig. 3 Distribution of cockroaches of both species around tree circumferences. Each triangle corresponds to the position of an individual adult male. Positions are displayed in two different frames of reference (*a* and *b*). *a* Positions relative to the upwind direction. *b* Positions relative to magnetic north

Gorton, 1980; different species and genera, Schal, 1982). *P. pennsylvanica* males were significantly higher up than *P. uhleriana* males (1.1 vs. 0.6 m averages; one-way *ANOVA*, $P<0.001$ for species).

Variability of Flow Direction at Tree Surfaces for Solitary Trees Variability in wind direction on a time scale of minutes is significantly affected by time of day, with evening wind direction being the least variable (Fig. 4). Note that our evening measurements were made during the courtship time of *Parcoblatta*. The standard deviation (circular) of wind direction of the minute-by-minute measurements over the 10-min intervals in the mornings averaged 17° ($N=4$ sets), in the afternoon, averaged 24° ($N=6$ sets), and in the evening, averaged 10° ($N=6$ sets) (ANOVA of wind variability with time of day, $P<0.01$, $N=16$).

Variability of Flow Direction at Tree Surfaces for Forest Trees Variability in wind direction in the forest was estimated by comparing upwind direction at the surface of trees early in the evening (8–9 P.M.) and later (between 1/2 and 2 1/2 hr later) at the same trees (10 trees at the forest edge and 10 more interior). The upwind direction at these surfaces typically did not change much during an evening: the average difference in upwind direction for individual forest trees was 23° for trees at the edge of the forest ($N=58$; averages of absolute differences) and 31° for the more interior trees ($N=36$; averages of absolute differences).

The variability of upwind direction at the surfaces of trees for a group of 10 trees was not affected by its location (edge vs. within forest) (Wilcoxon sign-rank test, $N=8$ sets of 20 trees, $P>0.05$). However, there was a small but significant difference in upwind direction at the surface of trees depending on whether the tree was at the edge of the forest or more interior: the average difference was 15° (Wilcoxon sign-rank test, $N=8$ sets of 20 trees, $P<0.027$), suggesting that the wind direction is shifted by the resistance of the vegetation as the air passes through the forest.

Wind speed was also affected by tree location (edge vs. within forest). The number of times during which flow was too slow to displace the boa feathers was significantly higher within the forest (wind not detectable in 44% of trials) compared to trees at the forest edge (wind not detectable in 17% of trials) (Wilcoxon sign-rank test, $N=17$ sets of 20 trees, $P<0.005$).

Detection of Pheromone Around a Cylinder in Controlled Flows Within our experimental conditions, sex pheromone was detected by at least some of the male moths, for any flow type, at any position or distance from the cylinder (Fig. 2). Across all trials, an average of 62% of the moths were able to detect pheromone (noted by wing fanning behavior; range

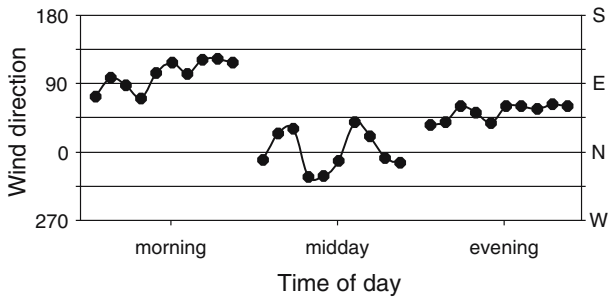


Fig. 4 Variability in upwind direction at a tree surface as a function of time of day. Shown is a representative set of measurements taken every min for 10 successive min at 3 different times during the day (starting at 8 A.M., 12 P.M., and 8 P.M.). Wind striking the tree surface at its northern-most point has a wind direction of "0" (east is 90, south is 180, west is 270)

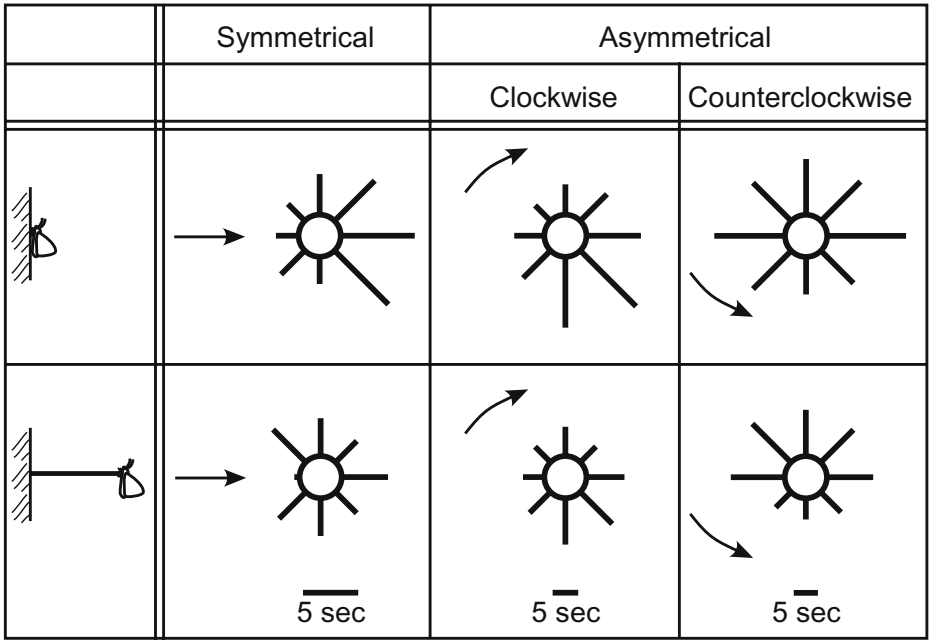


Fig. 5 Male moths at different positions around the cylinder wing at different times. Moths fanned whether the moths were located at the surface (*top row*) or 5 cm from the surface (*bottom row*). The *left column* illustrates the orientation of the moths when viewed from the side. Moths on the upwind areas tended to wing fan before moths on the downwind areas, for all flow types. The *lengths of the lines* represent the median of the wing fan onset times for all moths at that location (during the 30-sec observation period, the light correction factor has been applied as explained in the text; each column is drawn to a different time scale as indicated)

35–100% at any position for any flow/distance from cylinder combination). Male moths at the surface of the cylinder (0 cm) showed a slightly higher tendency to wing fan than males 5 cm from the surface (67 vs. 60%; chi-square $P=0.006$). There was a difference in the probability of pheromone detection at different positions around the cylinder in symmetrical flow for males positioned 5 cm from the cylindrical surface but not for males at the surface (0 cm) (chi-square $P<0.001$ for frequency of wing fanning by position for the symmetrical flow when 5 cm from surface, $P=0.5$ for 0 cm). Position around the cylinder did not affect the probability of pheromone detection in asymmetrical flow (chi-square $P>0.05$ for all four cases).

In almost all cases, males upwind on the cylinder fanned earlier than males downwind. For males in asymmetrical flow at both distances (0 and 5 cm) from the surface and males in symmetrical flow on the surface (0 cm), individuals on the upwind half of the cylinder fanned earlier than those on the downwind half (Mann–Whitney, $P_{Z,\ 2\text{-tailed}}<0.001$; Fig. 5). In symmetrical flow, the narrow pheromone plume closely followed the contour of the cylinder, so for moths 5 cm from the surface, only those directly upwind intercepted the plume as it approached the cylinder, whereas all others experienced a delay as the plume reached them by a combination of eddies and molecular diffusion. These eddies are sufficiently rapid that in this case (symmetrical flow, moths 5 cm from the surface), there was a marginally significant difference in fanning time between the front and back halves of the cylinder with males on the back half fanning slightly earlier on average than males on the front half (Mann–Whitney, $P_{Z,\ 2\text{-tailed}}<0.054$), although males at the single position

most upwind fanned the earliest (Fig. 5). Positions perpendicular to the flow were not included in the analysis for symmetrical flow because they are neither upwind nor downwind.

The median response time for moths in symmetrical flow was the same whether the moths were 0 or 5 cm from the surface (paired *t*-test by position $P=0.554$; Fig. 5). In the more erratic asymmetrical flows, moths positioned 5 cm from the surface responded more rapidly than moths positioned at the surface (paired *t*-test by position $P<0.032$ and $P<0.005$ for clockwise and counterclockwise flows, respectively). Pheromone detection, as denoted by the onset of wing fanning, occurred throughout the 30-sec observation period. No male moths fanned during the three controls. In addition, there was no evidence that a wing fanning male influenced the response of its neighbors by its wing fanning (by changing the probability or timing of their responses), similar to observations on wing fanning in gypsy moths by Elkinton et al. (1984).

Discussion

There are potential advantages of being on the upwind surface of a tree for an organism chemically sampling its environment. An organism there will usually intercept an airborne chemical signal earlier than an organism on the downwind surface, as we have shown here. In addition, an organism on the upwind surface will sample a chemical plume before it has undergone the additional mixing and dilution that usually occurs in the eddies downwind of the object. However, in practice, it may be difficult, if not impossible, for an organism already on the surface to locate the upwind side. Although air strikes the upwind surface, it also strikes the downwind surface as it swirls behind the object (Fig. 2), and therefore, an organism has ambiguous information about the ambient wind direction if it is walking along the surface (Brady et al., 1989, 1990). In contrast, a flying insect approaching a tree could land on either the downwind or the upwind surface, depending on whether it was flying upwind or downwind before landing. The effect of wind direction on landing patterns of flying insects has been investigated by Broce et al. (1991), who found that both stable flies, *Stomoxys calcitrans*, and house flies, *Musca domestica*, were more likely to land on the downwind side of cylinders. Landing in these areas offers increased protection from the wind, and the downwind landing preference was strengthened with increased wind speed (Broce et al., 1991).

We have measured the time delay in pheromone interception experienced by males on the downwind surface of cylinders for a range of slow flow types (Fig. 5). The magnitude of time delay will be a function of the air speed and the size of the tree. For slow air speeds typical of a forest, males on the upwind surface detected pheromone seconds to tens of seconds before the downwind males on the same cylinder. Particularly long delays are a result of the pheromone being returned to the surface after recirculating in an eddy. Whether seconds of difference confer an advantage to an organism on an upwind surface (compared to a downwind surface) may depend on the mating system of that species. For example, for competing organisms tracking a sex pheromone, the first to locate the pheromone-emitting individual could have a mating advantage. In addition, for blood-feeding insects such as tsetse flies, a delay in detection may cause difficulties in locating a host before that host moves away. Note that frequent wind shifting found in some habitats would diminish this prospective small upwind advantage. Willemse and Takken (1994)

have discussed the general difficulty of locating an upwind host by tsetse flies when the wind direction is shifting.

In addition to the velocity differences that exist between the upwind and downwind areas of an object, air moves more slowly next to an object's surface and diminishes to a velocity of zero at the surface (the "no-slip condition"). This slow flow near a surface can impact the behavior of organisms; for example, minute scale insects need to rear up on their hind legs to reach faster air flow farther from a leaf surface to take off in dispersal (Washburn and Washburn, 1984). Because of slowed air adjacent to a tree surface, an insect on a tree could project its antennae out into faster air flow to sample a larger volume of air per unit time and more rapidly detect pheromone. Using *B. mori* as pheromone detectors, we found that moths positioned 5 cm from the surface responded more rapidly on average than moths positioned at the surface, but pheromone was detectable at both of those distances. *Parcoblatta* cockroaches have long antennae (~2–3 cm), whereas *B. mori* moths' antennae are a tenth as long (~2–3 mm). By positioning the *Bombyx* moths both 0 and 5 cm from the surface, we encompassed a range potentially sampled by cockroaches and other forest insects. The flow pattern around a cylinder (Fig. 1) is an approximation for a flow pattern around a fairly cylindrical tree; the flow will also be affected by the roughness of the surface of the tree (e.g., bark texture).

The flow behavior depicted in Fig. 1 (eddies moving downwind or shed vortices) will be relevant for a broad range of tree sizes and wind speeds and can be predicted by the magnitude of a dimensionless number, the Reynolds number, $Re=uL/\nu$ (u=velocity of the air, L=tree diameter, ν=kinematic viscosity of the air; Brady et al., 1989; Vogel, 1994). Eddies that move downwind are predicted for the combination of tree sizes and wind speeds that result in $Re>40$ (Fig. 6), which includes the majority of natural conditions in a forest (Brady et al., 1989). The degree to which this predicted air flow pattern is relevant around trees in a forest depends, in part, on the wind variability. Note that if the wind is shifting direction frequently, the flow pattern will resemble Fig. 1 less closely, and there is less advantage and more difficulty for an organism to position itself upwind.

Our minute-by-minute measurements of wind direction at tree surfaces were significantly less variable in the evening than during the morning and midday (Fig. 4). The circular standard deviation was 10° in the evening, so about 65% of successive minute-by-

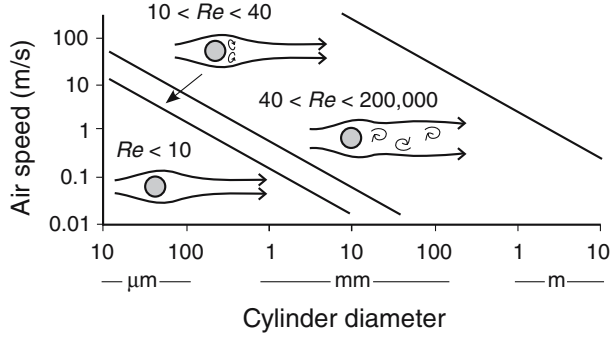


Fig. 6 Air flow patterns around a cylinder as predicted from fluid mechanics. The *air patterns* shown (for air approaching from the left) are expected for a cylinder in cross-flow for a wide range of air speeds and cylinder diameters (Vogel, 1994). The air flow conditions in this study were within the large interval represented by Reynolds numbers (Re) in the range $40<Re<200{,}000$, within which downwind eddies are expected. The kinematic viscosity of air is assumed to be 15×10^{-6} m^2/sec in the calculation of Re. See text for the equation to calculate Re. (Figure modified from Fig. 21.4 in Loudon, 2003)

minute measurements of upwind direction are within 10° (normal distribution approximation). Upwind direction measured at tree surfaces in the forest over longer intervals (at least 1/2 hr) during evenings differed only by an average of 26° (between the earlier and later measurements). Under these conditions, a stationary individual on the upwind half of a tree is likely to remain there for at least several minutes. Because of the lower variability in wind direction during evening hours, evening is the most advantageous time for pheromone-detecting individuals to position themselves on the upwind surfaces of trees, but this was not observed in the field for *Parcoblatta* males.

It is difficult to make direct comparisons of wind variability with other studies because the reported variability in wind direction will be strongly influenced by the sampling frequency (Brady et al., 1989) and whether the direction is measured against a tree surface or in the space between trees. However, the wind variability that we documented is similar to that reported by Elkinton et al. (1987) between trees in a coniferous forest, and much less than reported by Brady et al. (1989) adjacent to tree surfaces in a "mopane" woodland. Wind speed also has been reported to be slower and less variable in the evening than in morning or midday (Zöllner et al., 2004).

Our results indicate that pheromone interception occurs from all locations around a cylindrical object such as a tree, which may partially explain the even distribution of *Parcoblatta* males around trees during courtship periods, despite the relatively consistent upwind direction. It is unknown whether a detection advantage of seconds would affect the mating success of a *Parcoblatta* male. It is also possible that not all observed males were actively courting and that individuals on upwind and downwind surfaces were engaged in different activities (e.g., tracking females vs. resting), as is assumed for tsetse flies selecting upwind and downwind resting sites (Brady et al., 1989). The population density is sufficiently low that the observed distribution of cockroaches is unlikely to result from avoidance; usually only one cockroach was seen on a single tree, although two or three were found on occasion, and many trees had none.

The presence of neighboring trees is expected to impact the flow around a tree, in both magnitude and direction (Smith et al., 1972). The physical influence of trees on downwind flow has been considered for the orientation of diurnal forest insects, and in some cases, is more important than the visual stimulus provided by the tree (Wyatt et al., 1993, 1997; Foster and Ayers, 1995). We found that the magnitude of the wind speed was lower within the forest than at its edge. As a result of the slow wind speed in a forest, the flow pattern will be controlled largely by the spatial pattern of tree trunks, branches, and leaves, instead of by large-scale atmospheric processes (Smith et al., 1972). For example, air may move more rapidly through a natural channel offering less resistance to the wind than within adjacent denser areas of the forest. A difference in air flow speeds between channels and denser areas may induce slow areas of circulation and weak ephemeral eddies that we attempted to mimic in our experimental flow chamber ("asymmetrical" flows; Fig. 2 middle and bottom). Even in these complex asymmetrical flows, we found earlier wing fanning response times for individuals positioned on the upwind halves of the cylinders.

The use of wing fanning to infer pheromone detection has been an influential and useful technique for understanding the behavior of pheromone plumes traveling through the environment (Elkinton et al., 1984; Sanders, 1986; Charlton and Cardé 1990). The use of wing fanning as a proxy for pheromone interception requires appropriate experimental design to control for other factors that influence fanning. We observed that wing fanning may be initiated by environmental stimuli in addition to the presence of sex pheromone, such as a sudden increase in light intensity or air movement, so we avoided supplying such stimuli as much as possible. No males wing fanned during the controls. In addition, the

small light gradient in our experimental flow chamber resulted in a small but consistent difference in fanning response; we corrected for this difference as described above, and used both clockwise and counterclockwise gyres for our asymmetrical flow patterns (Fig. 2). Wing fanning may be affected by other factors, such as temperature (Elkinton et al., 1984).

The microhabitat selected by an organism will influence both the type and amount of information available to that organism because the characteristics of the environment will affect stimulus transmission (Dusenbery, 1992). Transmission of a chemical stimulus will be particularly dependent on air flow (or water flow for aquatic organisms) in the microhabitat (Atema, 1988; Murlis et al., 1992; Koehl, 2006). Organisms at different locations around the surface of an object will experience differences in flow, with upwind organisms usually intercepting a chemical signal earlier. Although the potential advantages of earlier interception from an upwind surface do not appear to be utilized by male *Parcoblatta* wood cockroaches in a forest, we have demonstrated that pheromone detection can occur at any position around a cylindrical object in a flow chamber, which could explain the even distribution of *Parcoblatta* males around trees.

Acknowledgments Custom-built solenoid valve controller for pheromone delivery was built by the Instrumentation Design Laboratory, University of Kansas. Grant support was provided to CL by the National Science Foundation IBN 9984475 and EPS-0236913 with matching support from the State of Kansas through Kansas Technology Enterprise Corporation. Support was provided to SF through NSF REU grant DBI-0353911 and to GLM through the KSR Small Grant program from the Kansas Biological Survey and the Entomology program at the University of Kansas. Derek Kellogg, Chelsea Johnson, and Eric Siegfried assisted with field observations, and Norm Slade with statistical analyses. The manuscript was improved by insightful comments from Derek Kellogg and two anonymous reviewers.

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J Chem Ecol (2007) 33:555–565
DOI 10.1007/s10886-006-9246-1

Sex Pheromone of *Lonomia obliqua*: Daily Rhythm of Production, Identification, and Synthesis

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Received: 11 September 2006 / Accepted: 18 December 2006 /
Published online: 25 January 2007
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Abstract The sex pheromone of *Lonomia obliqua* Walker (Lepidoptera: Saturniidae) was studied in the laboratory. All female calling occurred during the scotophase. Most females (70.6%) called first within 24 hr of eclosion. Calling varied with age of female, with older (5- to 6-day-old) females calling earlier in the scotophase and for longer durations than younger (0- to 1-day-old) females. The sex pheromone gland of 1- to 3-day-old virgin females was extracted during the calling peak. A Y-olfactometer bioassay showed significant attraction of males to a filter paper containing the female gland extract. Gas chromatographic-electroantennogram detection (GC-EAD) analysis of the extract indicated the presence of at least two possible pheromone components. Gas chromatographic-mass spectrometric analysis of the major GC-EAD-active peak indicated a hexadecenyl acetate; chemical derivatization indicated $\Delta 11$ unsaturation. Synthetic samples of (*E*)- and (*Z*)-11-hexadecenyl acetate were obtained by coupling 10-bromo-1-decanol and 1-hexyne, utilizing lithium chemistry. The comparison of the retention time of dimethyl disulfide derivatives of the natural compound, to those of synthetic chemicals, confirmed the natural compound as (*E*)-11-hexadecenyl acetate. The minor component was identified as the related alcohol, (*E*)-11-hexadecenol. The ratio of the two components in female extract was 100:35. Preliminary tests of males in a Y-olfactometer showed that their response to a mixture of the two compounds was not significantly different from that to gland extract.

Keywords Hemorrhagic caterpillar · Sex pheromone · (*E*)-11-Hexadecenyl acetate · (*E*)-11-Hexadecenol · Calling behavior

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Introduction

Lonomia obliqua Walker (Lepidoptera: Saturniidae) is one of the most important urban insects in Brazil due to the urticating spines on the larva. Contact with the toxin present in the spines can cause burns, bleeding, and, in worst cases, acute renal failure, hemorrhagic disorder, and death (Duarte et al., 1990). In Brazil, the distribution of the insect is concentrated in the southern states. Aspects of the biology and morphology of *L. obliqua* have been reported (Lorini, 1999, 2004; Lorini and Corseuil, 2001).

Compared to some families of Lepidoptera, there are relatively few studies on the identification of sex pheromones of Saturniidae. The sex pheromones of *Antheraea polyphemus* (Kochansky et al., 1975), *Nudaurelia cytherea* (Henderson et al., 1973), *Antheraea pernyi* and *Samia cynthia ricini* (Bestmann et al., 1989), and some species in the genera *Hemileuca* and *Saturnia* (McElfresh and Millar, 1999a,b,c; McElfresh et al., 2001a, b) are known.

The aim of the present study was to determine the effect of age on the calling behavior of *L. obliqua* females and to identify the sex pheromone of the species. Additionally, the behavioral responses of males to gland extract and to a blend of synthetic chemicals were also evaluated.

Methods and Materials

Insect Rearing Adults used in the experiments were obtained from caterpillars collected in the field in the northern region of the state of Rio Grande do Sul, Brazil. Insects were maintained in a controlled-environment room, with a mean temperature of 25±0.5°C, RH 65±15%, and a 13:11 (L to D) photoperiod. Larvae were allowed to feed on the leaves of *Platanus acerifolia* until pupation. Pupae were transferred into plastic containers (8×3 cm) lined with filter paper and maintained in an environmental chamber under the same conditions as for the larvae. The filter papers in the pupal containers were moistened daily. At the beginning of each scotophase, adults were collected and sexed according to the method of Lorini (1999).

Calling Behavior of Females After emergence, virgin females were transferred to a room at the same conditions as used for pupae. Females were placed in individual cages made of plastic tubes (PVC), measuring 15×10 cm, sealed on one side with transparent glass and on the other with nylon net. They were observed during the scotophase of each day until death. A tungsten lamp, covered with three layers of red cellophane paper and positioned approximately 80 cm from the cage, was used during observations of behavior. To determine the effect of age on calling, 65 virgin females were observed from the first to the sixth day after emergence, at 10-min intervals during each scotophase. From these observations, we obtained information about the time of onset of calling, the time spent calling, and the number of calling bouts.

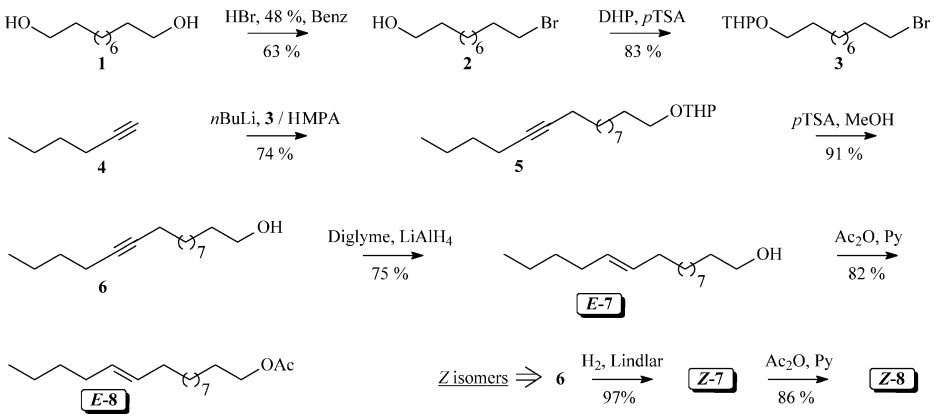
Extraction of Sex Pheromone from Glands Light pressure was applied to the terminal part of the abdomen of a female to expose the last abdominal segments on which the sex pheromone gland is located. The gland was dissected and extracted for 30 min in a 2-ml glass vial containing 0.5 ml of hexane. The extract was transferred to a clean vial, concentrated under a flow of argon, and stored at −20°C until use.

Analytical Procedures Coupled gas chromatographic-electroantenographic detector (GC-EAD) analysis was performed on male antennae with a system based on that of Leal et al. (1992, 1994). A Varian 3800 gas chromatograph, equipped with a DB-5 column (30 m× 0.25 mm×0.25 μm; Agilent Technologies, Santa Clara, CA, USA) and a splitless injector, was used in these analyses. The oven was programmed from 70°C (1 min hold) to 250°C at 7°C/min and kept at this temperature for 5 min. Gas chromatography-mass spectrometry (GC-MS) analyses were performed with a Varian 3800–Saturn 2000 GC-MS-MS ion trap detector, fitted with a DB-5 capillary column, under the same conditions as described above.

Synthesis High-grade reagents and solvents were used in the syntheses. Chromatographic purifications were carried out on silica gel 60, Merck, 230–400 mesh. Dimethyl disulfide (DMDS) derivatization was carried out according to the method of Vincenti et al. (1987). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 (400 and 100 MHz, respectively).

The starting material, 1,10-decanediol (**1**), was subjected to monobromination to yield 10-bromodecan-1-ol (**2**), which was subsequently protected with DHP to give ether **3**. The anion generated with *n*-BuLi from 1-hexyne (**4**) was alkylated with bromide **3**, affording compound **5**, which was directly deprotected to yield alcohol **6**, the direct precursor of the pheromones, because it possesses a triple bond at C-11 and functionality at C-1. The isomer (*E*)-11-hexadecenol (**7**) was obtained by reacting alcohol **6** with $LiAlH_4$, under reflux of diglyme, while the isomer (*Z*)-11-hexadecenol (**7**) was prepared by catalytic hydrogenation of the same compound **6**. The ^{1}H NMR analysis of the vinyl protons of the resulting compounds indicated an isomeric purity >97%. The isomers (*E*)- and (*Z*)-11-hexadecenyl acetate (**8**) were obtained by acetic anhydride acetylation of the corresponding alcohols (*E*)-**7** and (*Z*)-**7**, respectively (Scheme 1).

10-Bromodecan-1-ol (2) To a stirred solution of 1,10-decanediol (**1**) (5.2 g; 30.4 mmol) in benzene (100 ml), 48% aqueous HBr (5.0 ml) was added at room temperature. The reaction was refluxed for 24 hr, trapping water with a Dean-Stark apparatus. The mixture was successively washed with 1 M NaOH, 10% HCl, and brine. The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude oil obtained



Scheme 1 Synthesis of the components of the sex pheromone of *L. obliqua* and their (*Z*)-isomers

was distilled to give compound **2** in 63% yield (4.4 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.28–1.46 (m, 12H); 1.56 (quint, J=6.8 Hz, 2H); 1.65 (bs, 1H); 1.85 (quint, J=6.8 Hz, 2H); 3.41 (t, J=6.8 Hz, 2H); 3.63 (t, J=6.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 25.72, 28.15, 28.74, 29.37 (2C), 29.47, 32.75, 32.81, 34.00, 62.98. GC-MS *m/z* (%): 220/218 (M-18, 0.14), 190/192 (7.7), 162/164 (10.8), 148/150 (54), 97 (39.8), 83 (53.4), 69 (93.4), 55 (96.6), 43 (43.7), 41 (100).

1-(Tetrahydro-2'-pyranyloxy)-10-bromodecane (3) To a stirred solution of **2** (3.5 g; 15.25 mmol) in dry THF (15 ml), some crystals of *p*TSA and dihydropyran (1.65 ml; 18 mmol) were added. The reaction was stirred at room temperature for 4 hr and then washed with brine. The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Crude product **3** (83%, 3.9 g) was employed in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.28–1.46 (m, 12H); 1.50–1.62 (m, 6H); 1.68–1.75 (m, 1H); 1.81–1.89 (m, 3H); 3.35–3.42 (m, 1H); 3.43 (t, J=6.8 Hz, 2H); 3.48–3.53 (m, 1H); 3.73 (dt, J=6.8 and 9.6 Hz, 1H); 3.84–3.90 (m, 1H); 4.56-4.58 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 19.72, 25.52, 26.22, 28.17, 28.75, 29.37, 29.43 (2C), 29.75, 30.80, 32.83, 34.02, 62.35, 67.66, 98.85. GC-MS *m/z* (%): 320/322 (M, 0.38), 109 (0.9), 101 (8.61), 85 (100), 69 (8.40), 55 (16.5), 41 (23.1).

1-(Tetrahydro-2'-pyranyloxy)-11-hexadecyne (5) To a stirred solution of 1-hexyne (**4**) (2.34 ml; 20.2 mmol) in dry THF (10 ml), under argon, *n*-BuLi (6.0 ml, 2.0 M in hexane) was added dropwise at −78°C. The solution was slowly warmed to 0°C and stirred at this temperature for 40 min. The reaction was cooled again to −78°C, and compound **3** (3.0 g, 10 mmol) in dry HMPA (2.0 ml) was added and left for 1 hr. The mixture was warmed to 0°C and stirred for 50 min. The reaction was extracted with ethyl ether and washed with brine. The organic layer was separated, dried (Na_2SO_4), and concentrated *in vacuo*. The crude product was purified by column chromatography (silica gel, hexane to ethyl acetate 9:1), affording **4** in 74% yield (2.2 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.91 (t, J=6.8 Hz, 3H); 1.45–1.52 (m, 24H); 1.53–1.58 (m, 2H); 2.11–2.16 (m, 4H); 3.35–3.41 (m, 1H); 3.47–3.53 (m, 1H); 3.73 (dt, J=6.8 and 9.6 Hz, 1H); 3.84–3.90 (m, 1H); 4.56–4.58 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.65, 18.46, 18.77, 19.72, 21.95, 25.54, 26.27, 28.87, 29.19 (2C), 29.51 (2C), 29.57, 29.78, 30.81, 31.30, 62.32, 67.69, 80.19 (2C), 98.83. GC-MS *m/z* (%): 322 (M, 0.44), 280 (0.33), 265 (1.26), 207 (1.1), 165 (1.84), 121 (4.07), 101 (27.9), 85 (100), 81 (35.4), 67 (41.2), 55 (48.7), 41 (71.1).

11-Hexadecyn-1-ol (6) To a stirred solution of **5** (2.0 g, 6.2 mmol) in MeOH (10 ml), some crystals of *p*TSA were added. The mixture was stirred at room temperature for 4 hr. Methanol was removed, and the residue was diluted in ethyl ether and washed with saturated solutions of $NaHCO_3$ and brine. The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Crude product **6** (91%, 1.34 g) was employed in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (t, J=6.8 Hz, 3H); 1.25–1.51 (m, 21H); 2.12–2.16 (m, 4H); 3.63 (t, J=6.8, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.64, 18.46, 18.77, 21.94, 25.76, 28.86, 29.18 (2C), 29.44, 29.48, 29.57, 31.29, 32.82, 63.05, 80.19 (2C). GC-MS *m/z* (%): 164 (1.48), 149 (2.06), 135 (4.23), 121 (10.1), 110 (19.4), 96 (88.6), 81 (100), 67 (76.8), 55 (69.7), 41 (99).

(E)-11-Hexadecen-1-ol (7) A solution of compound **6** (1.5 g, 6.30 mmol) in diglyme (10 ml) was added dropwise to a suspension of $LiAlH_4$ (0.23 g, 6.30 mmol) in diglyme (10 ml) at room temperature. The resulting mixture was stirred at 130°C, under argon, for

5 hr. The reaction was quenched by the addition of aqueous NaOH, 0.1 mol/l (10 ml), followed by the addition of ether (50 ml). The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by column chromatography (silica gel, hexane to ethyl acetate 9:1), affording (*E*)-**7** in 75% yield (1.1 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (t, J=6.8 Hz, 3H); 1.25–1.40 (m, 19H); 1.53–1.61 (m, 2H); 1.94–1.99 (m, 4H); 3.65 (t, J=6.8, 2H); 5.37–5.40 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.97, 22.20, 25.75, 29.16, 29.44, 29.50, 29.56 (2C), 29.60, 29.66, 31.85, 32.29, 32.61, 32.83, 63.11, 130.32 (2C). GC-MS *m/z* (%): 240 (M, 0.09), 222 (5.23), 194 (1.87), 180 (0.92), 152 (3.0), 138 (7.23), 123 (14.0), 109 (24.4), 96 (64.5), 82 (92.3), 67 (55.4), 55 (100), 41 (75.0).

(Z)-11-Hexadecen-1-ol (7) To a stirred solution of **6** (0.5 g, 2.1 mmol) in MeOH (5 ml), 5% $Pd/BaSO_4$ (20 mg) and quinoline (two drops) were added. The mixture was stirred under hydrogen atmosphere (25 psi) in a Parr® apparatus for 4 hr and filtered on Celite®. The solvent was evaporated *in vacuo*, and the residue was extracted with ether and washed with 10% HCl, water, and brine. The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The product was purified by column chromatography (silica gel, hexane to ethyl acetate 9:1), affording (*Z*)-**7** in 97% yield (0.48 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (t, J=6.8 Hz, 3H); 1.24–1.39 (m, 19H); 1.53–1.60 (m, 2H); 1.97–2.04 (m, 4H); 3.64 (t, J=6.8, 2H); 5.33–5.36 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.97, 22.20, 25.75, 29.16, 29.44, 29.50,29.56 (2C), 29.60, 29.66, 31.85, 32.29, 32.61, 32.83, 63.11, 130.32 (2C). The MS data are the same as described for the (*E*)-isomer **7**. The retention times of compounds (*Z*)- and (*E*)-**7** were identical to that of the minor component of the natural pheromone.

(E)-11-Hexadecenyl acetate (8) To a stirred solution of pyridine (0.5 ml) and compound (*E*)-**7** (0.5 g, 2.1 mmol), acetic anhydride (0.4 ml, 4.0 mmol) was added, and the resulting mixture was stirred overnight at room temperature. The reaction was diluted in ethyl ether (20 ml) and washed with saturated $NaHCO_3$ solution, 10% HCl, and brine. The organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The product was purified by column chromatography (silica gel, hexane to ethyl acetate 9:1), affording (*E*)-**8** in 82% yield (0.47 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.89 (t, J=6.8 Hz, 3H); 1.22–1.40 (m, 18H); 1.55–1.65 (m, 2H); 1.96–2.02 (m, 4H); 2.05 (s, 3H); 4.05 (t, J=6.8, 2H); 5.37–5.40 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.02, 22.20, 25.93, 28.62, 29.15, 29.27, 29.50 (2C), 29.52 (2C), 29.66, 31.85, 32.29, 32.61, 64.67, 130.33 (2C), 171.22. GC-MS *m/z* (%): 282 (M, 0.09), 222 (15.2), 194 (2.90), 180 (1.82), 166 (5.30), 152 (5.44), 138 (13.45), 124 (23.8), 110 (33.7), 96 (82.9), 82 (100), 67 (56.4), 55 (78.8), 43 (81.8), 41 (61.2).

(Z)-11-Hexadecenyl acetate (8) In the same manner as for the (*E*)-isomer, the alcohol (*Z*)-**7** (0.2 g, 0.83 mmol) afforded the acetate (*Z*)-**8** in 86% yield (0.2 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.90 (t, J=6.8 Hz, 3H); 1.25–1.38 (m, 18H); 1.58–1.65 (m, 2H); 1.98–2.03 (m, 4H); 2.05 (s, 3H); 4.05 (t, J=6.8, 2H); 5.33–5.36 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.00, 21.02, 22.36, 25.92, 26.93, 27.20, 28.61, 29.28 (2C), 29.52 (3C), 29.77, 31.98, 64.67, 129.87 (2C), 171.24. The MS data are the same as described for (*E*)-**8**. The retention times of compounds (*Z*)- and (*E*)-**8** were identical to that of the major component of the natural pheromone.

Olfactometer Tests The behavior of males in the presence of the natural female pheromone was observed in a Y-olfactometer. In the first test, a glass olfactometer, with arms 10×

40 cm, was used. The bioassay was conducted during the scotophase at 25±1.0°C and 65±15% RH. The airflow, produced by a vacuum pump, was 2.5 l/min. A piece of filter paper, 2 cm^2, loaded with 5 μl (one female equivalent) of female gland extract, was placed at the extremity of one arm, and a filter paper with solvent applied (and left to evaporate) was placed in the other arm as a control. A virgin male was placed in the olfactometer, and which of the two arms he moved into was recorded; if he did not move into either arm, he was recorded as a nonresponder. Twelve replicates were performed. For the second test, 0.1 μg of a 1:0.35 mixture (on filter paper) of synthetic (*E*)-11-hexadecenyl acetate:(*E*)-11-hexadecenol was tested against a solvent blank. In the third test, the same synthetic blend as in the second test was tested against one female equivalent of extract. Ten replicates (each a single male) were performed for the last two tests.

Statistical Analyses Comparisons of the calling behavior on different days were made by ANOVA, followed by a Tukey highest significant difference test (Statistica and Stat Soft, Inc., 1999). The olfactometer responses were analyzed by using the R Statistical System (R Development Core Team, 2005). The choice experiment (gland extract vs. hexane) was tested with a GLM for Quasibinomial data using the logit link function (Crawley, 2005). Proportions were calculated by dividing the numbers of insects that chose a particular arm by the total insects tested. The analysis was carried out as follows. A full model was fitted, and significance was assessed by testing the change in deviance after the removal of a term from the model. After fitting the full model, the data were examined for overdispersion by dividing the residual deviance by the residual degrees of freedom to give the dispersion parameter; relatively large values of residual deviance suggest overdispersion and risk potential overestimation of the significance level (Crawley, 2002). To account for this, the residual deviance was rescaled by the dispersion parameter, and an *F* test was used to evaluate whether the removal of a term caused a significant increase in deviance. In addition, model fit was checked by examining the distribution of residuals. The validity of each analysis was verified with residuals analysis (Crawley, 2005). All significant differences are reported at $P<0.05$.

Results and Discussion

Virgin *L. obliqua* females exhibited calling behavior only during the scotophase. The behavior consisted of a female positioning with the wings totally opened, showing the end part of the abdomen, and exposing the ovipositor and pheromone gland. This compares to the work by Fornés and Hernández (2000) on *Hylesia metabus* (Lepidoptera: Saturniidae), who showed that females are active during crepuscular–nocturnal hours but not active during the photophase.

The mean age of females calling for the first time was 13.9±12.6 hr after emergence, with the range being 1.2–51.5 hr. The percentage of females that first called on the first day after emergence was 70.6%, on the second day, 27.8%, and on the third day, 1.6%. Among the females, 92.3% were observed to call once a day, 6.2% twice a day, and only 1.6% three times during the same day. Because not all female callings happened at the same chronological age, comparisons were made by "calling age" (Turgeon and McNeil, 1982).

Age-related data for onset and duration of calling are reported in Table 1. In general, females began calling earlier in the scotophase as they aged, with the mean onset of calling being significantly different between 1-day-old and 5- and 6-day-old females. Furthermore, older females tended to call for a longer duration than younger females. These age-related

Table 1 Mean onset time (hours into the scotophase) and mean time spent calling during the scotophase for different-age virgin females of *L. obliqua* (±SEM), (*N*=65)

Days	Mean Onset Time of Calling[1]	Time Spent Calling (hr)
1	6.0±0.2 a	3.20±0.21
2	5.35±0.2 ab	3.81±0.27
3	5.2±0.2 ab	4.26±0.30
4	5.25±0.2 ab	4.00±0.35
5	4.85±0.4 b	4.17±0.50
6	4.84±0.4 b	4.37±0.51

[1] Means in the column followed by the same letter are not significantly different (Tukey HSD test, $P<0.05$).

effects on calling probably ensure that older, unmated females will mate by attracting males earlier in the scotophase than younger females are capable of doing (Swier et al., 1977).

Sex pheromone glands from virgin females, 1- to 3-day-old, were extracted at peak calling (sixth–seventh hour into scotophase), and the extracts were tested in the olfactometer. A significantly greater percentage ($F_{1,10}=5.82$, $P=0.016$) of males were attracted to the arm containing the pheromone extract than to the control arm (Fig. 1). Responses of males in the olfactometer were similar to those observed for *Phyllocnistis citrella* (Gracillariidae) (Parra et al., 2002; Parra-Pedrazzoli et al., 2006) and suggested that the gland extracts elicit sexual attraction of males.

GC-EAD analysis of extract by using male antennae showed antennal responses to two components present in the gland extract (Fig. 2). The major EAD-active peak was at 25.31 min, whereas the minor one was at 23.51 min. The flame ionization detector showed the two compounds to be present in the extract at a ratio of 100:35. GC-MS analysis of the major EAD-active peak gave identifying features for a monounsaturated C16-acetate: a strong peak at *m/z* 43 and a peak at *m/z* 222, resulting from loss of acetic acid from the molecular ion *m/z* 282 (confirmed by chemical ionization measurement). The minor constituent was presumed to be the related alcohol because the mass spectrum showed a *m/z* of 222, suggesting loss of water from the molecular ion. The molecular weight was confirmed as 240 by chemical ionization.

Double-bond positions in the two components of the *L. obliqua* pheromone extract were determined by reaction with DMDS. GC-MS analysis of the resulting mixture showed the

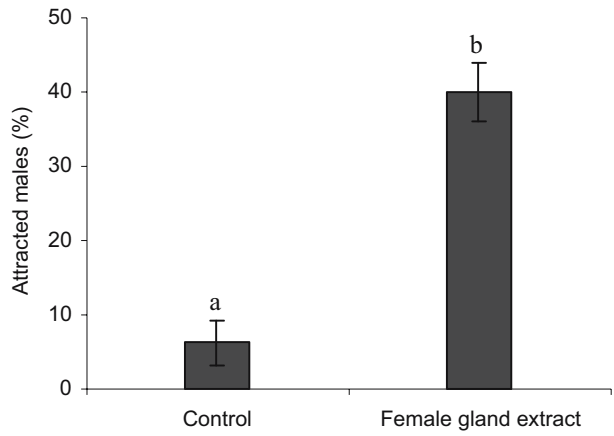


Fig. 1 Y-olfactometer test of *L. obliqua* to virgin female pheromone gland extract and a control. Different letters atop bars indicate significantly different means ($P<0.05$)

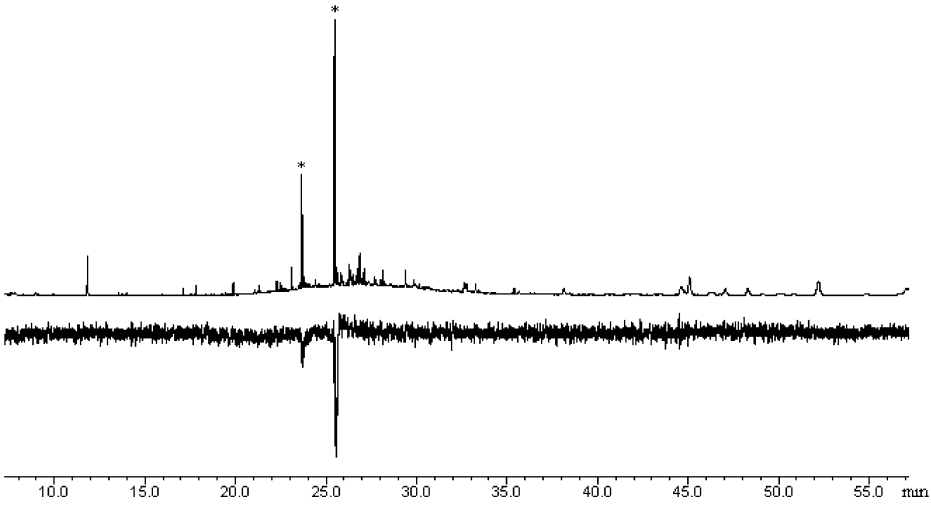


Fig. 2 Coupled gas chromatograph-electroantennogram detection (GC-EAD) analysis of female *L. obliqua* pheromone gland extract; the flame ionization detector response is above the EAD response. (*) = EAD-active peak

presence of the DMDS adduct of a Δ11-hexadecenyl acetate [*m/z* 376 (M^+) and major fragments at *m/z* 259 and 117] and of a Δ11-hexadecenol [*m/z* 334 (M+), *m/z* 217, and *m/z* 117] (Fig. 3).

To determine the geometry of the double bond of the compounds and to provide samples for laboratory and future field bioassays, we carried out a six-step synthesis of (*E*)- and (*Z*)-

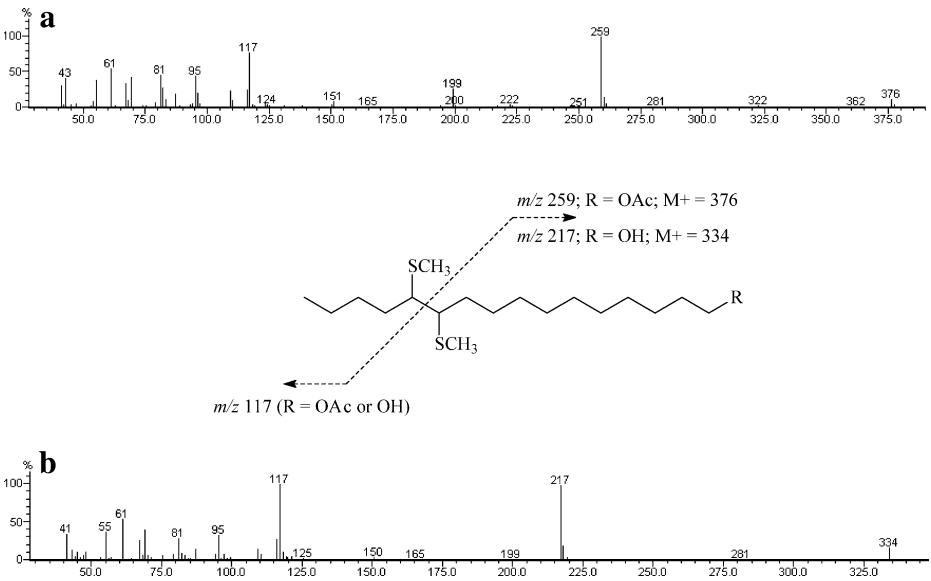


Fig. 3 Mass spectra of derivatives obtained by DMDS reaction with the two components of the pheromone gland extract of *L. obliqua*. The major product (A) and the minor (B) gave a fragmentation pattern that confirmed the location of the double bond at position 11

isomers of 11-hexadecenyl acetate (**8**) and 11-hexadecen-1-ol (**7**) based on the work of Kang and Park (1988) (see Scheme 1).

The mass spectra of synthetic alcohols **7** and acetates **8** were indistinguishable from those of the minor and major components of the natural pheromone, respectively. However, retention time data alone did not establish the geometry of the double bond because the isomers were poorly resolved on the DB-5 column. Therefore, all the synthetic isomers of **7** and **8** were converted to DMDS adducts, and the retention times of the synthetic adducts compared with those of the DMDS adducts of compounds in the pheromone extract. The addition of DMDS to double bonds for monounsaturated compounds is a stereospecific process, with the (*Z*)-isomer affording the *threo*-adduct and the (*E*)-isomer giving the *erythro*-adduct (Caserio et al., 1985). Even though adducts of regio-isomers show nearly identical EI-mass spectra, they typically have different retention times on capillary gas chromatography (Attygalle, 1998).

Almost baseline separation of (*Z*)- and (*E*)-11-hexadecenyl acetate–DMDS derivatives (34.7 and 35.1 min) was achieved by using the DB-5 column. The corresponding DMDS derivative in the pheromone extract had the same retention time as the (*E*)-isomer. DMDS adducts of (*Z*)- and (*E*)-hexadecenol were also easily resolved (32.4 and 32.8 min, respectively). The corresponding adduct in the pheromone extract had the same retention time as that of the (*E*)-isomer (Fig. 4). Based on these results, the two compounds were identified as (*E*)-11-hexadecenyl acetate (major component) and (*E*)-11-hexadecenol (minor component).

In the preliminary olfactometer trial that tested the synthetic blend of the two compounds versus a solvent blank, 60% of the males moved into the arm with the synthetic blend, whereas only 10% moved into the solvent blank arm (the remainder were nonresponders). A further preliminary olfactometer trial that tested the synthetic blend against female extract showed that 40% of the males moved into the arm with the synthetic blend and 30% into the arm with the extract. Although preliminary in nature, these experiments suggest that the two-component synthetic blend does attract male *L. obliqua*. Together, our data indicate that a mixture of (*E*)-11-hexadecenyl acetate and (*E*)-11-hexadecenol forms the sex pheromone of *L. obliqua*. This is the first report of these compounds as sex pheromone components of a saturniid moth.

In summary, we have described the periodicity of the calling behavior of *L. obliqua* females and identified their sex pheromone. Field experiments are now in progress, which aim to try to develop a program to monitor populations and prevent problems caused by this pest.

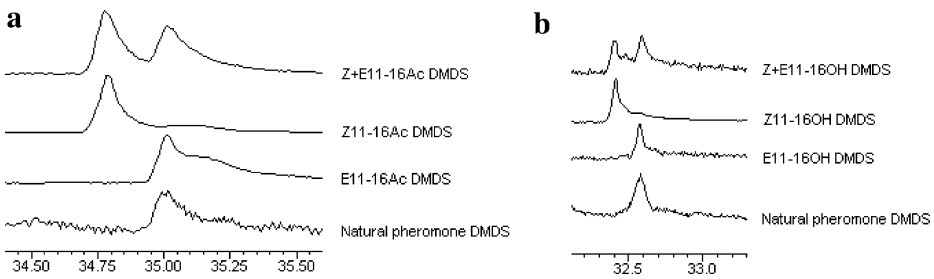


Fig. 4 Determination of the double-bond geometry of (A) 11-hexadecenyl acetate (Z+E11-16Ac) and (B) 11-hexadecenol (Z+E11-16OH) of the two sex pheromone components of *L. obliqua* using DMDS derivatization

Acknowledgments We thank the International Foundation for Science—Sweden, the Organization for the Prohibition of Chemicals Weapons—The Netherlands, CNPq, Fundação Araucária, and the Embrapa/Prodetab for the financial support.

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J Chem Ecol (2007) 33:567–578
DOI 10.1007/s10886-006-9223-8

Oviposition Responses of Gravid Female *Culex quinquefasciatus* to Egg Rafts and Low Doses of Oviposition Pheromone Under Semifield Conditions

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Received: 25 August 2006 / Accepted: 9 November 2006 /
Published online: 25 January 2007
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Abstract Semifield experiments were conducted to study the oviposition of gravid *Culex quinquefasciatus* females in response to one or 10 egg rafts, or 3.0 μg of synthetic oviposition pheromone, (-)-(*5R,6S*)-6-acetoxy-5-hexadecanolide, a dose equivalent to 10 egg rafts. These treatments were added to the small bowls filled with either hay infusion or water in a small (0.3-m spacing) or a large-square design (3.4-m spacing). Oviposition choice was more pronounced in the "small square" assays. Mean number of egg rafts laid in response to a single egg raft in an oviposition jar filled with hay infusion was significantly greater than with all other treatments. When the oviposition pheromone dose was increased from one to 10 rafts or when 3.0 μg synthetic oviposition pheromone were dispensed on a floating receptacle, synergistic effects were observed between the oviposition pheromone and the hay infusion at both distances. This study is the first demonstration that the amount in a single raft induces oviposition of gravid *Cx. quinquefasciatus* females under semi-natural conditions.

Keywords - (-)-(*5R,6S*)-6-acetoxy-5-hexadecanolide · *Culex quinquefasciatus* · Culicidae · Egg raft · Hay infusion · Mosquito · Oviposition · Pheromone

Introduction

Gravid mosquitoes use a combination of sensory modalities to find and determine the suitability of water-containing sites for egg deposition. Habitat-related odors and a pheromone associated

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with the egg rafts can influence the propensity of gravid female *Culex* mosquitoes to oviposit (for review, see McCall, 2002). After the original experiments with natural egg rafts (Osgood, 1971; Osgood and Kempster, 1971; Bruno and Laurence, 1979), the pheromone was identified as (-)-(*5R,6S*)-6-acetoxy-5-hexadecanolide (Laurence and Pickett, 1982). Several synthesis routes have been developed, usually resulting in a mixture of two (Laurence and Pickett, 1982) or four stereoisomers (Dawson et al., 1990; Olagbemiro et al., 1999; Michaelakis et al., 2005).

The egg aggregation activity of the synthetic pheromone has been confirmed in laboratory assays (Laurence and Pickett, 1982, 1985; Laurence et al., 1985; Hwang et al., 1987; Otieno et al., 1988a; Dawson et al., 1990; Mordue et al., 1992; Blackwell et al., 1993; Millar et al., 1994; Olagbemiro et al., 2004) and in field trials (Otieno et al., 1988b; Mboera et al., 2000a,b; Olagbemiro et al., 2004). The antipode does not induce *Culex quinquefasciatus* (Say) oviposition (Laurence and Pickett, 1985; Hwang et al., 1987) and, presumably, does not hinder the activity of the synthetic pheromone (Laurence and Pickett, 1982; Dawson et al., 1990; Olagbemiro et al., 1999, 2004). However, no studies have directly compared the synthetic racemic mixture with pure pheromone compound (or raft), which is needed to validate the latter suggestion.

Oviposition bioassays with *Culex* species traditionally have been performed in small screened cages. In such a setup, the placement of a single egg raft in one of two oviposition bowls led to a significant tendency for the females to oviposit in that bowl (Bruno and Laurence, 1979), and the threshold quantity of synthetic oviposition pheromone dispensed from a floating receptacle was determined to be 0.02 μg (Laurence and Pickett, 1985), which is about 1/15 the amount of pheromone present in a single egg raft (Laurence and Pickett, 1982). Hwang et al. (1987) reported a 50-fold reduction of the threshold quantity of pheromone needed when the pheromone was applied directly on the water surface. A dose of 100 μg of pheromone on a floating receptacle reduced egg raft deposition (Blackwell et al., 1993).

No dose-response experiments in the field have been reported for the oviposition pheromone; without exception, field trials have been carried out by treating oviposition sites with effervescent tablets laced with 5 mg of the active pheromone (Otieno et al., 1988b; Mboera et al., 2000a,b; Olagbemiro et al., 2004), an equivalent of over 16,000 egg rafts. Previous studies demonstrated that the oviposition pheromone, (-)-(*5R,6S*)-6-acetoxy-5-hexadecanolide induces oviposition in gravid *Cx. quinquefasciatus* females under particular conditions in the laboratory (small cages) and field (high doses of pheromone), but do not establish whether a oviposition response is induced by the pheromone under more natural conditions.

In the present study, gravid *Cx. quinquefasciatus* females were provided oviposition sites with natural doses of oviposition pheromone (one raft, 10 rafts, or equivalent doses of synthetic oviposition pheromone, (-)-(*5R,6S*)-6-acetoxy-5-hexadecanolide) at two different distances in outdoor large screened cages. Additional four-choice assays were performed to validate the inactivity of the pheromone's antipode (+)-(*5S,6R*)-6-acetoxy-5-hexadecanolide by testing it against the solvent or in a direct comparison with the synthetic oviposition pheromone. In two-choice bioassays, the preference of females for laying eggs in an oviposition site containing 10 rafts or an equivalent dose of the synthetic oviposition pheromone in a racemic mixture with the antipode was determined. We hypothesized that a female's choice of where to deposit her eggs will be more pronounced in the assays where the stimuli were placed near each other than at a distance. Two-choice bioassays, a common experimental design, often provide increased discrimination over the same treatments presented singly, because in the former case responders can simultaneously sample two alternatives. Such simple choices may not present themselves in nature, however, where responders are more likely to have to make "take it or leave" it choices (Wagner, 1998; Cardé, 2005). The latter comparisons may provide a more realistic measure of the selective forces dictating selection of an oviposition site in nature.

Methods and Materials

Mosquitoes *Culex quinquefasciatus* originated from Riverside County, CA, USA and were maintained in a climate room (27±2°C, 70±3% RH) with a 16:8 L/D cycle including a 1-hr dusk-and-dawn period provided with one 25-W incandescent light bulb. Larvae were reared on an artificial diet (3:1, rat chow/yeast mixture) in plastic trays (4 L). Each day, pupae were collected and allowed to emerge in 30×30×30 cm screen cages (Bioquip®, Rancho Dominguez, CA, USA) and provided with a 10% sucrose solution. A week to 10 d after emergence, mosquitoes were offered the opportunity to feed overnight on live 1- to 5-d-old white leghorn chicks, *Gallus gallus domesticus* L., (UCR animal use protocol #A-0404016-2). The following morning, the mosquito cage was transferred to a greenhouse with a natural photoperiod. The greenhouse was temperature controlled (23±4°C, 56±15% RH). Six to eight d after blood feeding, gravid females were collected in 400-ml cardboard containers (20 per container) and used in the experiments.

General Experimental Conditions Bioassays were conducted outdoors in tent-shaped screen cages ($h \times w \times l$, 2×4×4 m, COS-1313S Quest), consisting of a roof and four screened sides with flaps at the bottom. Long plastic pipes (5 cm i.d.) filled with sand for ballast were placed on the flaps to prevent released mosquitoes from escaping. Cages were arranged in a square (20×20 m) in the shade of trees (Chinese wingnut, *Pterocarya stenoptera*) at Agricultural Operations of the University of California at Riverside, from August 5 to November 28, 2005. Each cage contained either four (Experiments 1–5) or two (Experiment 6) oviposition jars. An oviposition jar consisted of a plastic container (175 ml, sidewall 5.5 cm, top diameter 9.5, bottom diameter 5.8 cm), in which a small glass "shell" vial (12×50 mm) was placed. The bottom of the glass vial was secured to the bottom of the containers with putty (QuakeHold, Trevco, CA, USA). The outer surface of the container was spray-painted black (NOW spray paint, Sherwin-Williams Co., OH, USA).

The oviposition jar (black container and glass vial) was filled with 100 ml of either hay infusion or water (details below). The glass vial of the oviposition jar received one of the oviposition pheromone related stimuli (OPRS) or a control treatment (see below). In the four-choice bioassays (Table 1: Experiments 1–5), the oviposition jars were arranged in either a small square (0.3×0.3 m) at the center of the cage ("near") or in a large square (3.4×3.4 m) at 0.3 m from the corners of the cage ("distant"). In the two-choice bioassay (Table 1: Experiment 6), the two oviposition jars were placed in opposite corners of either the small or large square. In each experiment, bioassays were replicated twice each night for four nights (N=8), with 20 gravid females per replicate at both distances. Experiments commenced an hour before sunset and ended approximately at 09:00 the next morning, when the number of egg rafts laid in each jars were counted and the females were recollected and removed from the cages. Different groups of mosquitoes and fresh stimuli were used in all tests.

Stimuli Hay infusion was prepared with the formula used by the Mosquito Control Research Laboratory in Parlier, CA, USA (University of California, Davis) as an oviposition medium in gravid traps: 5.6 g of dried and shredded Bermuda grass and 0.34 g of bakers yeast per 2.5 L water. The hay infusion in a 3.8-L glass jar was placed in a hood in the laboratory. After 7 d, the infusion was filtered through a cloth to remove the grass fragments and used in the experiments.

Synthetic samples of the mosquito oviposition pheromone (MOP), (5*R*,6*S*)-6-acetoxy-5-hexadecanolide and its antipode, (5*S*,6*R*)- 6-acetoxy-5-hexadecanolide, were provided by

Table 1 Set up of semifield oviposition experiments with *Culex quinquefasciatus*

Experiment[a]	2005	Oviposition Jars[b]			
	I		II	III	IV
1	Aug. 16–23	Hay infusion+ 1 raft	Hay infusion+ 1 artificial raft	Water+1 raft	Water+ 1 artificial raft
2	Aug. 5–18	Hay infusion+ 10 rafts	Hay infusion+ 10 artificial rafts	Water+10 rafts	Water+ 10 artificial rafts
3	Oct. 14–25	Hay infusion+ 3.0 μl OP[c]	Hay infusion+ 3.0 μl hexane	Water+3.0 μl OP	Water+ 3.0 μl hexane
4	Oct. 25–29	Hay infusion+ 3.0 μl AP[d]	Hay Infusion+ 3.0 μl hexane	Water+3.0 μl AP	Water+ 3.0 μl hexane
5	Nov. 14–18	Hay infusion+ 6.0 μl racemic[e]	Hay infusion+ 3.0 μl OP+ 3.0 μl hexane	Hay infusion+3.0 μl AP+3.0 μl hexane	Hay infusion+ 6.0 μl hexane
6	Nov. 18–24	Hay infusion+ 10 rafts	Hay infusion+ 3.0 μl OP	–	–

[a] All experiments were replicated with oviposition jars arranged in a small square at the center of the tent ('near') and in a large square ('distant') $N=8$.

[b] Oviposition jars contained either 100 ml of hay infusion or water.

[c] OP=1,000 ppm oviposition pheromone (−)-(*5R,6S*)-6-acetoxy-5-hexadecanolide.

[d] AP=1,000 ppm antipode, (+)-(*5S,6R*)-6-acetoxy-5-hexadecanolide.

[e] AP=1,000 ppm racemic mixture of oviposition pheromone and antipode.

Bedoukian Research Inc. Samples were analyzed by gas chromatography (6890 Series GC, Agilent Technologies, Palo Alto, CA, USA) to determine chemical purity. Hexane solutions (see below) were injected in splitless mode with the inlet and FID at 250°C and separated on a HP-5 column (25 m×0.32 mm; 0.25 μm). The oven was operated at 100°C for 1 min, increased to 250°C at a rate of 10°C/min, and held at the final temperature for 5 min. Under these conditions, both pheromone and antipode appeared at 18.14 min. Each sample gave a single peak in addition to the solvent front. Next, we determined the enantiomeric purity of the two stereoisomers by gas chromatography equipped with a chiral column, Chiraldex GTA (25 m×0.25 mm; 0.125 μm; Astec, Whippany, NJ, USA), which was operated at constant temperature, 175°C. MOP appeared at 92.78 min, whereas the retention time of the antipode was 91.62 min. The enantiomeric excess of (5*R*,6*S*)- and (5*S*6*R*)- 6-acetoxy-5-hexadecanolide were estimated as >99.5% and >99%, respectively, thus indicating a high enantiomeric purity.

The chemical analysis above indicated that samples prepared with the same nominal concentrations gave peaks with different areas, possibly because of residual organic solvents in the synthetic samples and/or experimental error in sample preparations. To generate samples with exactly the same concentrations, we prepared 10 μg/μl hexane solutions (10,000 ppm) of MOP and its antipode and dilute them with distilled hexane into 5, 1, and 0.1 μg/μl. These samples (1 μl of each concentration) were injected in three replicates, and the mean area for each concentration was plotted vs. concentrations to generated linear curves ($R^2>0.99$), with different slopes. The highest concentration of the samples generating large FID areas were adjusted, the adjusted sample was diluted, and the procedure was repeated until the slope of the MOP and antipode samples were identical. After confirming that the 1 μg/μl (1,000 ppm) samples of the two semiochemicals gave

nearly the same FID responses, the samples were used for behavioral studies. Racemic samples were prepared by adding equal volumes of the two stereoisomers.

The following oviposition pheromone-related stimuli (OPRS) were used: i. one egg raft, ii. 10 egg rafts, iii. 3.0 μg (-)-(*5R,6S*)-6-acetoxy-5-hexadecanolide (oviposition pheromone), iv. 3.0 μg (+)-(*5S,6R*)-6-acetoxy-5-hexadecanolide (antipode), v. 0.6 μg racemic mixture (3.0 μg oviposition pheromone and 3.0 μg antipode). Egg rafts, laid by gravid *Cx. quinquefasciatus* females the night preceding the experiment, were transferred onto the glass vials of the oviposition jars, already filled with hay infusion or water, just before the start of the experiment. To control for visual cues of the rafts used in Experiments 1 and 2, equivalent numbers and sized artificial rafts, made of pieces of black closed cell foam rubber (Tight Fit Foam Weather Strip, W.J. Dennis, IL, USA), were used in the control oviposition jars. Doses of 3.0 μl of oviposition pheromone or the antipode in hexane (1,000 ppm) or 6.0 μl of a racemic mixture of the two compounds in hexane (1,000 ppm) were applied onto a microscope cover glass (12 mm diameter) cut in half. After the solvent had evaporated, the cover glasses were wrapped individually in aluminum foil, transported to the field site, and transferred with the treated side upward onto the glass vials of the oviposition jars, already filled with hay infusion or water, just before the start of the experiment. Similarly treated cover glasses with equivalent doses of hexane served as controls. Each stimulus was tested twice at each corner of the squares and each stimulus combination was tested twice in each of the four cages.

Experiments The study consisted of six experiments (Table 1). In Experiment 1, gravid females were introduced into cages containing two oviposition jars with 100 ml of hay infusion and two with 100 ml of water. Two jars (one with hay infusion and one with water) received a single egg raft, and two jars received an artificial egg raft (see above). Experiments 2–4 were set up similarly, except for using 10 rafts, 3.0 μg of oviposition pheromone or 3.0 μg of the antipode (or their appropriate controls), respectively, instead of one raft as the OPRS. In Experiment 5, gravid females were introduced into cages containing four oviposition jars with 100 ml of hay infusion each. Jars received either 3.0 μg of oviposition pheromone, 3.0 μg of the antipode, 6.0 μg of the racemic mixture, or an equivalent amount of hexane. In Experiment 6, gravid females were introduced into cages containing two oviposition jars, each with 100 ml of hay infusion. Jars received either 10 egg rafts or 6.0 μg of the racemic mixture of the oviposition pheromone and antipode. In Experiment 6, there were no controls for the visual cues presented by the rafts or cover glasses.

Statistical Analyses Under the assumption that a gravid female deposits her eggs in a single raft (Clements 1992), egg raft counts correspond with the numbers of females ovipositing. Because in some cases data were not normally distributed, data were analyzed by nonparametric tests. Within jar arrangement (distant or near), factorial experiments (Experiments 1–5) were each subjected to Friedman's method for randomized blocks, followed by Wilcoxon signed ranks test to compare differences between treatment means (N=8) (Sokal and Rohlf, 2005). The results of the two-choice bioassay (Experiment 6) were analyzed by using the Wilcoxon signed ranks test (N=8). The results of all experiments together were subjected to Wilcoxon signed ranks test to determine significant differences between the numbers of egg rafts laid in the replicates with the jars arranged near each other with the distant ones; for each night, the numbers of egg rafts laid in the two replicates of the former were summed and paired with the total of rafts laid in the two replicates of the latter (N=24, Experiments 1–6 replicated for four nights).

Results

For all four-choice bioassays (Experiments 1–5), Friedman's method for randomized blocks indicated highly significant ($P<0.005$) effects of the treatments on the numbers of rafts laid in the oviposition jars. Results of the follow-up test (two-tailed Wilcoxon signed ranks test) to compare differences between treatment means are shown in Figs. 1 and 2. Experiment 1 revealed that the mean number of egg rafts laid by gravid *Cx. quinquefasciatus* females in response to a single raft floating in an oviposition jar filled with hay infusion was significantly greater than the mean numbers laid in any of the three other oviposition jars present when the jars were placed near each other, but not when they were placed 3.4 m apart. In the latter case, the mean numbers of rafts in the two jars filled with hay infusion containing either a floating single egg raft or an artificial raft were not significantly different ($Z=1.439$, $P=0.150$, two-tailed Wilcoxon signed ranks test). In Experiment 2, the mean number of egg rafts laid in response to 10 rafts floating in an oviposition jar filled with hay infusion (Fig. 1) was greater than the mean number laid in response to 10 artificial rafts floating on hay infusion when the oviposition jars were placed near each other ($Z=2.521$, $P=0.012$) and when placed at a

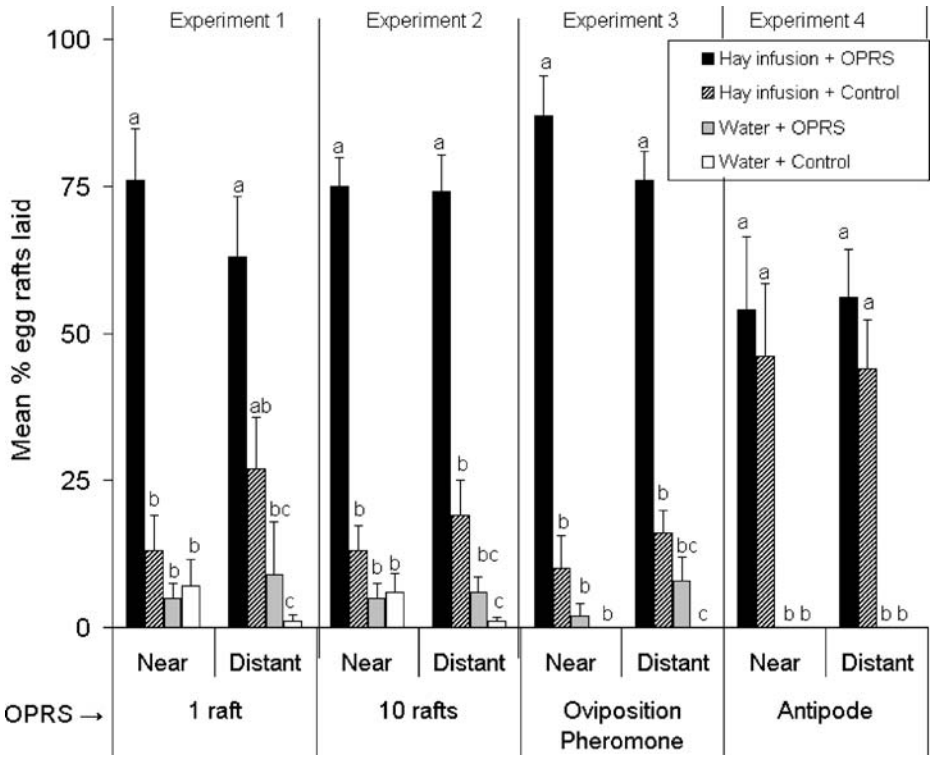


Fig. 1 Oviposition of gravid females of *Culex quinquefasciatus* in eight four-choice bioassays in outdoor tents (Experiments 1–4). Stimuli were presented either near (0.3 m) or distant (3.4 m) from each other within a tent. Vertical bars represent standard errors ($N=8$). Within each bioassay, statistical analysis was carried out using Friedman's method for randomized blocks, followed by Wilcoxon signed ranks tests (different letters denote significant differences at $P<0.05$, two-tailed). OPRS=Oviposition Pheromone Related Stimulus, Control=appropriate control for the OPRS used (see Table 1), Oviposition Pheromone=3.0 μl 1,000 ppm oviposition pheromone, Antipode=3.0 μl 1,000 ppm antipode, Racemic=6.0 μl 1,000 ppm racemic mixture of oviposition pheromone and antipode

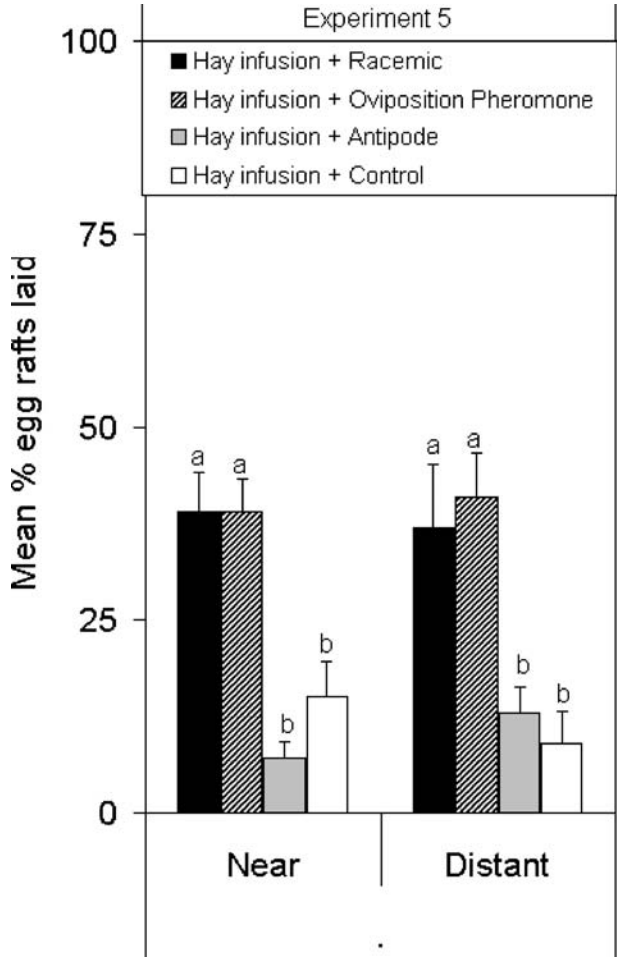


Fig. 2 Oviposition of gravid females of *Culex quinquefasciatus* in two four-choice bioassays (Experiment 5) in outdoor tents. Stimuli were presented either near (0.3 m) or distant (3.4 m) from each other within a tent. Vertical bars represent standard errors (N=8). Within each bioassay, statistical analysis was carried out using Friedman's method for randomized blocks, followed by Wilcoxon signed ranks tests (different letters denote significant differences at $P<0.05$, two-tailed). Racemic=6.0 μl 1,000 ppm racemic mixture of oviposition pheromone and antipode, Oviposition Pheromone=3.0 μl 1,000 ppm oviposition pheromone, Antipode=3.0 μl 1,000 ppm antipode, Control=6.0 μl hexane

distance (Z=2.527, P=0.012). Similar results were found when using 3.0 μg of oviposition pheromone (Experiment 3: Z=2.527, P=0.012; Z=2.533, P=0.011), but not when using 3.0 μg of the antipode (Experiment 4: Z=0.680, P=0.496; Z=0.681, P=0.496) as the OPRS. In all four preceding experiments (Experiments 1–4), the mean numbers of rafts laid in response to OPRS on water was not significantly different ($P>0.05$) from its control on water, at either distance. For both distances in Experiment 5 in which all oviposition jars were filled with hay infusion (Fig. 2), no significant differences in mean number of rafts were found between oviposition jars containing 6.0 μg of racemic mixture of the oviposition pheromone and the antipode, and an equivalent amount of pure oviposition pheromone (near: Z=0.213, P=0.872; distant: Z=0.845, P=0.012) or between 3.0 μg of the antipode and the control with only the solvent (near: Z=0.816, P=0.414; distant: Z=1.005, P=0.315). In the two-choice bioassays of Experiment 6 (Fig. 3), the mean number of rafts laid in response to 6.0 μl of racemic mixture was not significantly different, from the mean number laid in response to 10 rafts in hay infusion (near: Z=1.693, P=0.090; distant: Z=1.160, P=0.246) at either distance.

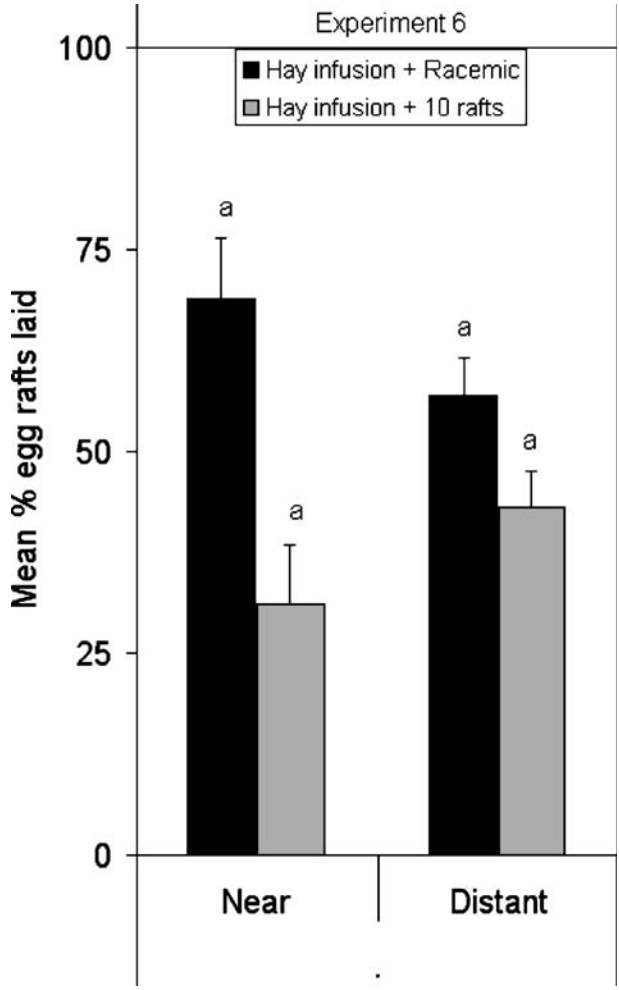


Fig. 3 Oviposition of gravid females of *Culex quinquefasciatus* in two two-choice bioassays (Experiment 6) in outdoor tents. Stimuli were presented either near (0.3 m) of distant (3.4 m) from each other within a tent. Vertical bars represent standard errors (N=8). Within each bioassay, statistical analysis was carried out using Wilcoxon signed ranks tests (different letters denote significant differences at $P<0.05$, two-tailed). Racemic=6.0 µl 1,000 ppm racemic mixture of oviposition pheromone and antipode

The mean numbers of rafts laid per night in the replicates with the jars placed near each other were lower than in those when they were placed distant from each other (Table 1, $Z=3.987$, $P<0.001$, two-tailed Wilcoxon signed ranks test).

Discussion

These oviposition studies were conducted outdoors under natural photoperiod and weather conditions, and with a moderately large experimental space to permit natural orientation behavior of the mosquitoes to occur. Our results confirmed laboratory studies (Bruno and Laurence, 1979) in that female *Cx. quinquefasciatus* differentiated between suitable oviposition sites with a single raft and nearby sites without an egg raft, and showed that this trend, although not significant, persisted when the distances between the oviposition sites were increased from 0.3 to 3.4 m. For an odor cue from an egg raft to evolve into an aggregation/oviposition pheromone, the presence of multiple egg rafts should improve

survival/reproduction of larvae from both the first and subsequently laid rafts. Presumably, such aggregation generally lessens the rate of predation on larvae, and this benefit outweighs the cost of pheromone production on female fecundity (McCall, 2002). Our study is the first demonstration of an effect of a single raft on the oviposition of gravid *Cx. quinquefasciatus* females under seminatural conditions. The priming effect of the first egg raft was enhanced by others subsequently laid in that bowl (Clements, 1999), but that is also expected to occur in the field (Reiskind and Wilson, 2004). When the stimulus was increased from one to 10 egg rafts, females showed a distinct preference for the hay infusion jars with the rafts at both distances tested.

The results of the bioassays with 3.0 μg pure synthetic oviposition pheromone were comparable with those of its natural equivalent (10 rafts); aggregation of egg rafts in the oviposition sites containing the oviposition-related stimulus. No significant differences were found between the numbers of rafts in the jars containing hay infusion with or without the antipode (Experiments 4 and 5, Figs. 1 and 2) or those with the pure oviposition pheromone or the racemic mixture (Experiment 5, Fig. 2), which confirmed that the antipode is not active in inducing or reducing oviposition by *Cx. quinquefasciatus* females (Laurence and Pickett, 1982, 1985; Hwang et al., 1987; Dawson et al., 1990; Olagbemiro et al., 1999, 2004). The direct comparison of day-old natural rafts with equivalent doses of synthetic oviposition pheromone in a racemic blend on a floating glass slide (Experiment 6, Fig. 3) revealed that despite differences in the mode of pheromone presentation, their effects on oviposition of *Cx. quinquefasciatus* were comparable. When oviposition sites were placed near each other, the synthetic pheromone tended to receive greater number of rafts than the natural pheromone source, but this difference was not significant.

This is the first report comparing the effect of the synthetic oviposition pheromone emanating from an artificial source with its natural counterpart. Aggregation of egg rafts was obtained with low doses (3.0 μg) of synthetic oviposition pheromone (pure or in racemic mixture) on a floating receptacle in our semifield trials. In previous field trials, high doses (5 mg) of active oviposition pheromone were applied onto the water surface by means of effervescent tablets (Otieno et al., 1988b; Mboera et al., 2000a,b; Olagbemiro et al., 2004). Increased oviposition activity was observed in the oviposition sites several days after the high initial dose of the synthetic oviposition pheromone (Otieno et al., 1988b; Mboera et al., 1999). Although the residual activity was not determined in our study, the results are promising for using low pheromone in field trials in less developed countries where the cost of the synthetic pheromone is an issue.

Oviposition jars containing water, irrespective of the presence of a floating oviposition pheromone-related stimulus (Experiments 1–4), received low numbers of rafts. This suggests that the role of the pheromone may be secondary to that of site-derived odors (Millar et al., 1994), although pheromone and kairomones act synergistically in our study and others (Otieno et al., 1988a; Mordue et al., 1992; Mboera et al., 2000a,b). Alternatively, contact components present in the oviposition medium used might play an important role in the acceptance or rejection of an oviposition site by the female. In our study, as in many other oviposition bioassays (Blackwell et al., 1993; Isoe and Millar, 1995; Olagbemiro et al., 1999, 2004; Mboera et al., 2000a; Michaelakis et al., 2005), only the overall effect of the pheromone and the hay infusion on oviposition was scored (egg raft count) without an analysis of the behavioral segments leading to this endpoint. Other bioassays, such as two-choice olfactometers (Osgood and Kempster, 1971), video recordings (Pile et al., 1991), sticky-screen assays (Isoe and Millar, 1995), and gravid traps (Mboera et al., 2000b) have been used in an attempt to distinguish the behavioral events leading to oviposition. However, in many cases the behavior of mosquitoes is affected by the bioassay design. We

scored egg rafts deposited on a known suitable oviposition substrate (Gjullin et al., 1965) or in water to compare the effects of various pheromone-related stimuli floating in a seminatural setting.

In Experiments 1–3, the number of egg rafts in the hay infusion jar with the OPRS was significantly greater than all three other nearby jars, which in turn were not significantly different. The latter was not the case in the replicates with the distant jars. Although this and the result of Experiment 6 support our hypothesis that the female's choices were more pronounced in the replicates with the oviposition sites near each other than those at a distance, the difference was more subtle than anticipated.

The total number of egg rafts deposited varied widely between the experiments that were conducted between August 5 and November 24 (Table 2). This large variation in oviposition activity was expected to be at least partly caused by the changing weather conditions over the 4-mo experimental period (the maximum daily temperature ranged from 18 to 35°C and the minimum relative humidity ranged from 7 to 67%). Unexpectedly, the total number of egg rafts deposited in a bioassay depended largely on the proximity of the oviposition jars. In all but one experimental night (Table 1, night 5), more rafts were laid in the replicates with the jars distant from each other than those nearby (Table 1). This phenomenon was probably an artifact of the experimental setup and may have been caused by differences in the average distance between the females and the jars rather than in the distance between the jars themselves. Most females rested on the screened sites of the cages

Table 2 Total number of egg rafts laid by *Culex quinquefasciatus* females in the replicates with the oviposition jars near and distant from each other

Experiment	Night	Egg Rafts	
		Near[a]	Distant[a]
1	1	8	8
	2	10	21
	3	17	19
	4	20	32
2	5	36	31
	6	16	23
	7	12	26
	8	31	35
3	9	19	29
	10	7	9
	11	11	14
	12	4	12
4	13	2	15
	14	9	16
	15	8	22
	16	8	19
5	17	15	33
	18	28	31
	19	10	18
	20	20	26
6	21	12	27
	22	8	13
	23	15	27
	24	15	20
Total no.[b]		341	526
Mean %±SE [c]		35.5±4.2 a	54.8±4.0 b

[a] Total number of two replicates per night with 20 females released in each.

[b] In 24 nights, a total of 960 females were released for each distance.

[c] N=24. Means followed by a different letter are significantly different at P<0.001, two-tailed Wilcoxon signed ranks test.

immediately after release from the center of the cage and were recovered from between the folds of the flaps at the bottom corners of the cage on the following morning. Thus, females may have had a higher probability of encountering suitable jars in the treatments with the jars at the corners of the cages than those with the jars arranged in a small square at the center of the cage.

Three possible explanations are suggested for the number of females ovipositing being clearly affected by distance, whereas their choice for an oviposition site seemed only marginally influenced. Females may fly along the edge of the cage in search of a suitable oviposition site without selecting the one first encountered, suggesting that females make a distinction between hay infusion sites based on the oviposition pheromone detected at a distance of a meter or so. Alternatively, females may fly along the edges of the cage and, upon encountering the pheromone at close range, land and/or oviposit. Another possibility is that females visit and land at several oviposition sites before deciding where to oviposit.

The oviposition pheromone thus could function as an attractant (Osgood and Kempster, 1971; Pile et al., 1993) in the former, and as an arrestant (Pile et al., 1991, 1993) and/or stimulant of oviposition in the second situation, or potentially a combination of both in the latter. Although organic infusions appear to function both as an attractant and oviposition stimulant for *Cx. quinquefasciatus* females (Ikeshoji, 1966a, b), further research is necessary to elucidate the modes of action of the oviposition pheromone for it to be effectively utilized in gravid traps. In particular, it will be useful to determine if these odors induce upwind anemotaxis or some form of chemotaxis in still air (collectively termed "attraction"), changes in velocity (orthokinesis), landing, or oviposition. Endpoint measurements such as egg deposition do not establish how orientation maneuvers influence oviposition.

Acknowledgment We thank Prof. W. Walton for providing the *Cx. quinquefasicatus* colony, S. Cockerham facilitating use of the field site at Agricultural Operations, UCR, Prof. J. Millar for the use of his greenhouse for maintaining mosquito colonies, and S. Raoufian for assistance in maintaining the mosquito colony. Dr. S. McElfresh is thanked for his advice on numerous occasions and Dr. M. Cooperband for her comments on an earlier version of the manuscript. We are grateful to Bedoukian Research Inc., Danbury, CT, USA for providing synthetic samples of the mosquito oviposition pheromone and its antipode. This research was supported by a grant from National Institutes of Health (# 1-UO1-A10058267).

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J Chem Ecol (2007) 33:579–589
DOI 10.1007/s10886-006-9221-x

(*Z*)-11-Hexadecenal and (3*Z*,6*Z*,9*Z*)-Tricosatriene: Sex Pheromone Components of the Red Banded Mango Caterpillar *Deanolis sublimbalis*

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Received: 17 July 2006 / Accepted: 3 November 2006 /
Published online: 31 January 2007
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Abstract The sex pheromone of the red banded mango caterpillar, *Deanolis sublimbalis* (Lepidoptera: Crambidae), a serious pest of the mango *Mangifera indica* (Anacardiaceae) in India and Southeast Asia and a recent invader into northern Australia, has been identified. Three candidate compounds were identified from pheromone gland extracts of female moths, using gas chromatography (GC), GC-electroantennographic detection and GC-mass spectrometric analyses, in conjunction with dimethyldisulfide derivatization. Field bioassays established that both (*Z*)-11-hexadecenal (*Z*11-16:Ald) and (3*Z*,6*Z*,9*Z*)-tricosatriene (3*Z*,6*Z*,9*Z*-23:Hy) were required for attraction of male *D. sublimbalis* moths, and 1,000 μg of a 1:1 mix of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy was more attractive to male moths than

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s10886-006-9221-x and is accessible for authorized users.

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caged virgin females. However, the binary blend was only attractive when the isomeric purity of the monounsaturated aldehyde was >99%, suggesting that the (*E*)-isomer was inhibitory. Although (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH) was tentatively identified in gland extracts, the addition of this compound to the binary blend did not increase the numbers of moths captured. The pheromone can now be used in integrated pest management strategies.

Keywords Red banded mango caterpillar · *Deanolis sublimbalis* · Odontiinae · Crambidae · Mango pest · (*Z*)-11-hexadecenal · (3*Z*,6*Z*,9*Z*)-tricosatriene · Synergistic sex pheromone components · Behavioral antagonist

Introduction

The red banded mango caterpillar *Deanolis sublimbalis* Snellen (Lepidoptera: Crambidae) is a serious pest of mango *Mangifera indica* (Anacardiaceae) in India and many regions of Southeast Asia (Golez, 1991; Waterhouse, 1998; Sujatha and Zaheruddeen, 2002). Damage is caused by the distinctively marked larvae boring into the flesh and kernel of mango fruitlets, allowing secondary infection to develop, which renders the fruit unsuitable for market or consumption (Golez, 1991; Waterhouse, 1998). Infestation levels of *D. sublimbalis* can exceed 40% in commercial mango crops (Tipon 1979 cited by Golez, 1991). Krull (2004) reported infestation levels of ca 4–23% at four sites in the Central Province of Papua New Guinea (PNG) with infestation on some trees being as high as 77%.

The range of *D. sublimbalis* extends from India east into Burma, China, Taiwan, and south into Thailand, the Philippines, Brunei, Indonesia, and PNG (Waterhouse, 1993; Li-Ying et al., 1997). Recently, this moth has been found on the tip of Cape York Peninsula in far north Queensland, Australia on isolated feral and noncommercial street and domestic mango trees (Foulis, 2000). Although the incursion of *D. sublimbalis* into Australia has so far been contained using strict quarantine measures (Foulis, 2000), the possibility of spread into the southern commercial growing areas threatening the Australian $100 million mango industry (AMIA, 2006) cannot be ruled out. Consequently, there has been an urgent need to develop sensitive monitoring methods to detect any southern spread.

Our objective was to identify the sex pheromone of *D. sublimbalis.* We report, here, the conclusive identification of one compound and tentative identification of two further compounds in pheromone gland extracts of *D. sublimbalis* and the evaluation of their biological activities in field bioassays in PNG.

Methods and Materials

Insects Infested mango fruitlets were collected from a mango orchard at Tahira (09.5157° S, 147.2876° E), Central Province, PNG, and kept in plastic containers until pupation of larvae. Larvae exited the fruit to pupate in a layer of sawdust in the bottom of the containers. Pupal cocoons were placed in sealed plastic containers (Lily® 35-ml portion cups with lids; Huhtamaki Henderson Ltd., Auckland, NZ) for shipment by air freight to HortResearch, Lincoln, New Zealand. Adult moths were allowed to emerge in a secure room in the Landcare Research quarantine facilities at Lincoln, and each sex was kept separately in 40 cm^3 humidified polycarbonate plastic boxes, at 25°C and 75% RH, under a 12:12 hr light:dark reverse photoperiod. Moths were provided with 10% sucrose solution.

Females had their pheromone glands excised and extracted, whereas males were used for coupled gas chromatography-electroantennographic detection (GC-EAD) analyses. Male *D. sublimbalis* have pronounced black tufts on their mid-tarsi, and an abdomen that extends beyond the hind wings, characters which aided sex determination.

Pheromone Gland Extracts All pheromone gland extracts were taken from 24- to 72-hr-old actively calling females, 2–3 hr into the scotophase. Excised pheromone glands were placed in 50 µl *n*-hexane in a liquid-nitrogen-cooled 0.5-ml conical vial. After all glands had been excised, the vial and its contents were brought to room temperature and left for 5 min before the supernatant was transferred to a 1.1-ml conical glass vial. Extracts were stored at −80°C until analysis. To quantify pheromone titer per female, the pheromone glands of 11, 15, and 17 females were extracted in 50 µl aliquots of *n*-hexane containing 50 ng of (*Z*)-10-pentadecenal (*Z*10-15:Ald) internal standard, as described above. The supernatant from each of these extracts was then concentrated under a stream of argon to 10 µl, and a 1-µl aliquot was analyzed by GC-mass spectrometry (GC-MS).

Chemicals *Z*10-15:Ald, (*Z*)-11-hexadecenal (*Z*11-16:Ald), (*E*)-11-hexadecenal (*E*11-16:Ald), (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH), and (*E*)-11-hexadecen-1-ol (*E*11-16:OH), were purchased from Pherobank, Wageningen, The Netherlands (all ≥97% isomerically pure by GC analysis). *Z*11-16:Ald of high isomeric purity (>99%) was also obtained from Pherobank for use in field bioassays. (3*Z*,6*Z*,9*Z*)-Tricosatriene (3*Z*,6*Z*,9*Z*-23:Hy; ca 95% isomerically pure) used for comparative chromatographic and spectrometric analyses was synthesized at HortResearch, Palmerston North. In addition, 3*Z*,6*Z*,9*Z*-23:Hy (>99% isomerically pure) was supplied by J. M. Chong (University of Waterloo, Ontario, Canada) for additional comparative analyses and for field bioassays.

Synthesis of (3Z,6Z,9Z)-tricosatriene Synthesis of (3*Z*,6*Z*,9*Z*)-tricosatriene is described in the online supplement at (see electronic supplementary material).

Gas Chromatography-Electroantennographic Detection Pheromone gland extracts of *D. sublimbalis* were analyzed by GC-EAD using a Varian 3800 gas chromatograph, equipped with both polar and nonpolar columns, coupled to an EAD Recording Unit (Syntech Research and Equipment, Hilversum, The Netherlands). For details of the preparation of moth antennae and the setup of associated equipment for GC-EAD analyses, see Gibb et al. (2006). Extracts were analyzed on DB-5 (30 m×0.25 mm i.d.×0.5 µm film; Agilent Technologies, Palo Alto, CA, USA) and BPX-70 (30 m×0.25 mm i.d.×0.25 µm film; SGE, Victoria, AU) columns, with 1:1 split outlets. Helium was used as carrier gas (1 ml min^{-1}), and injections were splitless for 0.6 min. Injector and detector temperatures were 220 and 300°C, respectively, and the GC temperature program was 80°C for 1 min, 10°C min^{-1} to 240°C, hold for 30 min. Kováts retention indexes (KIs) (Kováts 1965; Marques et al., 2000) were calculated for the compounds that elicited antennal responses.

Gas Chromatography-Mass spectrometry Pheromone gland extracts of *D. sublimbalis* and *Z*11-16:Ald, *E*11-16:Ald, *Z*11-16:OH, *E*11-16:OH, and 3*Z*,6*Z*,9*Z*-23:Hy standards were analyzed by GC-MS on VF-5 ms (30 m×0.25 mm i.d.×0.25 µm film; Varian Inc., Walnut Creek, CA, USA) and BPX-70 (30 m×0.25 mm i.d.×0.25 µm film; SGE) columns, using a Varian 3800 gas chromatograph, with a 0.6-min splitless injection (injector 220°C), coupled to a Varian 2200 MS. The helium flow was 1 ml min^{-1} with the transfer line at 250°C, and the ion

trap temperature at 200°C. The GC temperature programs were 1 min at 80°C, 10°C min^{-1} to 240°C, and hold for 15 min (VF-5 ms column), or 1 min at 80°C, 10°C min^{-1} to 130°C, hold 3 min, 5°C min^{-1} to 190°C, and hold for 20 min (BPX-70 column).

Dimethyldisulfide Derivatizations For dimethyl disulfide (DMDS) derivatizations of *Z*11-16:Ald, *E*11-16:Ald, *Z*11-16:OH, and *E*11-16:OH standards (10 ng in 20 μl of *n*-hexane) and a 10-female *D. sublimbalis* gland extract, we followed the procedure described by Buser et al. (1983) and Leonhardt and DeVilbiss (1985). Approximately 50 μl of DMDS and 5 μl of iodine solution (60 mg of I_2 in 1 ml of Et_2O) were added to the solution of interest in a 1.8-ml glass vial sealed with a Teflon-lined cap and were reacted at 40°C for 15 hr. The reaction was quenched with 50 μl of a 5% aqueous solution of $Na_2S_2O_3$, and the organic layer was dried with anhydrous Na_2SO_4, transferred to a clean conical vial, and concentrated with a stream of argon to less then 5 μl. A 1-μl aliquot was analyzed by GC-MS on the VF-5 ms column with the temperature program described previously (injector 250°C; 8 min solvent delay). The adducts from the (*E*) and (*Z*)-hexadecenals and the hexadecen-1-ols, although having identical spectra, were easily separated.

Field Bioassays Field bioassays were carried out in PNG during the 2005 mango season, in street trees in Port Moresby (trial A) and in mature orchards at Tahira (trials B and C; "Kensington Pride" mango) and the Pacific Adventist University (PAU), Port Moresby (09.4073° S, 147.2781° E) (trial D; main mango cultivars were "Kensington Pride", "Glenn" and "Irwin"). Green delta traps (Clare et al., 2000) were attached to trees at a height of ca 2.5 m, with at least 20-m spacings between traps and at least 20-m spacings between replicates in all trials. Trap positions within each replicate were re-randomized, and sticky inserts were replaced each time traps were checked. Test compounds, in ratios established from GC-MS analysis of *D. sublimbalis* gland extracts, were loaded onto red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA).

In trial A (July 19 to August 4), the attraction of *D. sublimbalis* males to various combinations of the three candidate compounds was tested. Traps were checked three times at intervals of 4–8 d. Treatments were (1) blank control, (2) 1000:0:0, (3) 0:1000:0, (4) 0:0:1000, (5) 500:500:0, (6) 1000:0:50, and (7) 500:500:50 μg loadings of *Z*11-16:Ald, 3*Z*,6*Z*,9*Z*-23:Hy, and *Z*11-16:OH (*N*=3).

Trial B (August 16 to September 27) was a repeat of trial A, but included caged virgin females as an additional treatment (treatment 8). Traps were checked 14 times at intervals of 1–5 d. Female moths were replaced every 3–5 d and supplied with 10% sugar solution applied to cotton dental rolls. There were three replicates.

Trial C was a dose response trial based on treatment 5 from trials A and B and was set up from September 27 to December 21. Traps were checked 18 times at intervals of 2–6 d, except for the last occasion when traps were left for 13 d because of low catches during that period. Treatments were (1) blank control, (2) 10, (3) 100, (4) 1000, and (5) 3000 μg doses of a 1:1 mix of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy (*N*=6).

In trial D (September 29 to November 6), the attraction of *D. sublimbalis* males to a 1000 μg dose of the 1:1 mix of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy was compared, in which *Z*11-16:Ald was either >99% (treatment 1) or ca 97% (treatment 2) isomerically pure (*N*=6). Traps were checked five times at intervals of 7–10 d.

Statistical Analyses Only treatments that trapped moths were included in the statistical analyses. Because there was some variation in the number of days between trap checks, the

response variate used in the analyses was the average number of moths caught per day. For trials A and B, a mixed model was fitted to the response variate, with treatment as the fixed effect, and date as the random effect.

For trial C, a mixed model was fitted to the response variate, with lure dose, assessment date and its interactions as fixed effects, and assessment date within replicates as random effects. Assessment date was included as both random and fixed effects to ascertain any systematic effects of assessment date with changes in lure dose (fixed effects) over and above the variability in the response variate due to assessment date (random effects).

Both models assumed that the error terms were normally and independently distributed, with zero mean and constant variance. The response variate was transformed, before analysis, using the square root transformation, to ensure that the model assumptions were met. Data were analyzed by analysis of variance (ANOVA) using GenStat Release 8.1 (VSN International Ltd., UK). Means separation tests for trials A and B were carried out using Fisher's protected least significant difference (LSD) at the 5% significance level. For trial C, means were separated with Tukey's honest significant difference (HSD) at the 5% significance level, to adjust for any false differences. Model adequacy checks were performed by examining histograms, normal probability plots, and scatter plots of the residuals.

Results

Identification of Pheromone Components In GC-EAD assays, male *D. sublimbalis* antennae responded repeatedly to three compounds in female moth pheromone extracts (Peaks 1–3; Fig. 1).

These compounds were identified as *Z*11-16:Ald, 3*Z*,6*Z*,9*Z*-23:Hy, and *Z*11-16:OH, as follows. The calculated KIs of compound 1 (1809 and 2373 on the DB-5 and BPX-70 columns, respectively) suggested the presence of a C_{16} monounsaturated aldehyde (Marques et al., 2000), and the retention times were identical to those of the *Z*11-16:Ald standard. Analysis by GC-MS provided further proof that compound 1 was *Z*11-16:Ald, with good retention time and mass spectral matches with the standard. Furthermore, GC-MS analysis of the DMDS-derivatized extract showed an adduct with a molecular ion at *m*/*z* 332 (11%) and diagnostic ions at *m*/*z* 117 (52%, $[C_5H_{10}SCH_3]^+$) and 215 (100%, $[OC_{11}H_{20}SCH_3]^+$), indicating the addition of DMDS to a double bond at position 11. We also noted that male *D. sublimbalis* antennae responded equally well to the *E*11-16:Ald standard (Peak b, Fig. 1; KIs of 1809 and 2348 on the DB5 and BPX-70 columns, respectively), even though this stereoisomer was not detected in gland extracts.

The mass spectrum of compound 2 [*m*/*z* 318 (2%, M^+), 262 (3%), 135 (14%), 121 (14%), 108 (28%), 93 (37%), 79 (100%), 67 (52%), 55 (22%) and 41 (41%)] and its elution order before tricosane on the VF5-ms column (KI 2272), suggested the nonconjugated C_{23} triunsaturated hydrocarbon, 3*Z*,6*Z*,9*Z*-23:Hy (Underhill et al., 1983; Millar, 2000). This was confirmed by matching retention times on the DB-5 and BPX-70 columns with those of a standard (KIs of 2270 and 2431, respectively) and by matching the mass spectrum to that of a standard. In addition, male *D. sublimbalis* antennae responded similarly to compound 2 and to the 3*Z*,6*Z*,9*Z*-23:Hy standard (Fig. 1), although the antennal response to the compound was relatively small compared with the response to the other pheromone components. We also observed that antennae of males responded to impurities in the synthetic 3*Z*,6*Z*,9*Z*-23:Hy including an unknown compound, and to isomers of 3*Z*,6*Z*,9*Z*-23:Hy (Peaks a, c, and d; Fig. 1). While it was not possible as part of this study to verify

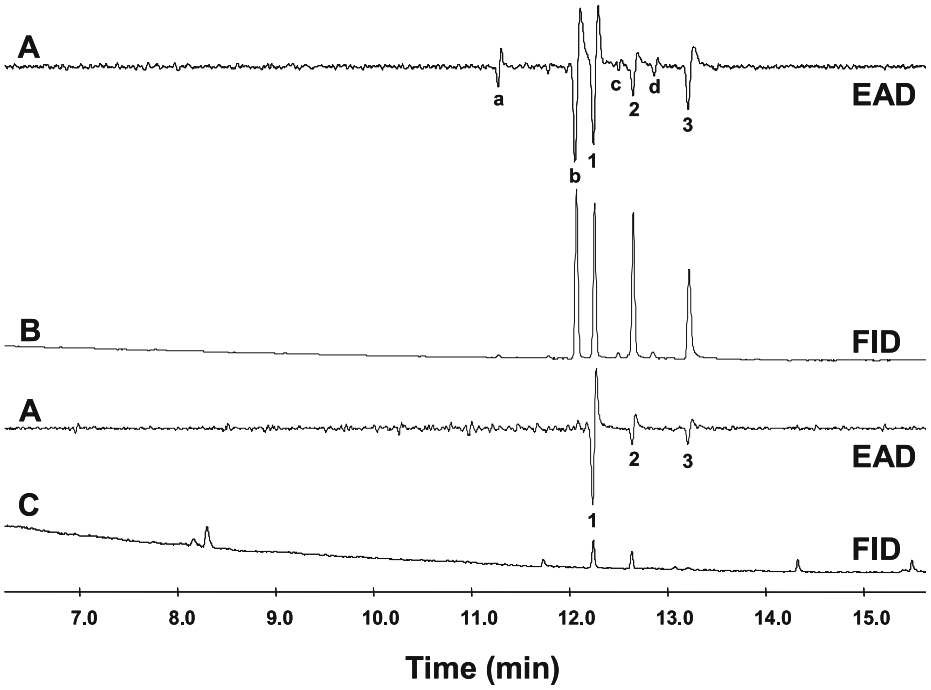


Fig. 1 Coupled gas chromatogram-electroantennograms of (**A**) *Deanolis sublimbalis* male antennae responding to (**B**) synthetic compounds (5 ng), and (**C**) insect-produced compounds in a *D. sublimbalis* female pheromone gland extract, eluting from a BPX-70 column. **1**=(*Z*)-11-hexadecenal, **2**=(3*Z*,6*Z*,9*Z*)-tricosatriene, and **3**=(*Z*)-11-hexadecen-1-ol. Spikes **a**, **c**, and **d** are antennal responses to an unknown compound and two unknown isomers of the C_{23} triunsaturated hydrocarbon respectively, in the synthetic (3*Z*,6*Z*,9*Z*)-tricosatriene. Spike **b** is the antennal response to synthetic (*E*)-11-hexadecenal

that all the double bonds of the natural tricosatriene have a *Z* configuration due to the small amount of material available, the results of the comparative analyses by GC and GC-MS and the biological activity of 3*Z*,6*Z*,9*Z*-23:Hy in GC-EAD and field bioassays strongly suggest the *Z*,*Z*,*Z* isomer. Therefore, we have tentatively identified compound 2 as 3*Z*,6*Z*,9*Z*-23:Hy.

We confirmed the double bond position and geometry of compound 3 to be *Z*11-16:OH by matching its retention times on the DB-5 and BPX-70 columns (KIs of 1865 and 2518, respectively) with those of the *Z*11-16:OH standard (Marques et al., 2000). Male *D. sublimbalis* antennae also responded well to *Z*11-16:OH (Fig. 1). Although compound 3 was a relatively minor component in extracts and not always detectable by GC-MS, we were nevertheless able to obtain an adequate spectrum from a composite extract of 36 female *D. sublimbalis* glands, which matched that of the standard. However, we were not able to verify the structure of compound 3 using DMDS derivatization because of co-elution and the small amount of material in the derivatized gland extract.

Analyses of three replicates of *D. sublimbalis* gland extract by GC-MS on the VF5-MS column revealed that *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy were present at a ca 1:1 ratio. The amount of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy per *D. sublimbalis* pheromone gland was calculated to be 237±37 and 247±97 pg (mean ± SEM), respectively. The proportion of

*Z*11-16:OH in *D. sublimbalis* pheromone gland extracts, compared with *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy, was ca 5% (*N*=3) by GC-EAD analysis.

Field Bioassays The results of the field bioassays established that both *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy were necessary and sufficient to attract male *D. sublimbalis* moths (Fig. 2). Analysis of the data from blend trials A and B indicated significant effects for treatment ($P<0.018$ and <0.001, respectively). In both trials, the blank controls or the lures loaded with the individual compounds were not attractive to male *D. sublimbalis*. Although the binary blend of *Z*11-16:Ald and *Z*11-16:OH (treatment 6) attracted a few male moths (*N*=4) in trial A, this same mix was not attractive at the Tahira mango orchard (trial B). However, at both sites, the binary blend of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy (treatment 5), and the ternary blend of *Z*11-16:Ald, 3*Z*,6*Z*,9*Z*-23:Hy, and *Z*11-16:OH (treatment 7) attracted moths (Fig. 2). In addition, at the mango orchard where we compared the synthetic compounds with caged virgin females, both treatments 5 and 7 were more attractive to male moths than were females. Although treatments 5 and 7 attracted similar numbers of moths in trial A, at the Tahira mango orchard (trial B), treatment 5 was more attractive to male moths than treatment 7.

In trial C, we tested a range of doses of the 1:1 blend of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy. Effects of lure dose ($P<0.001$), assessment date ($P<0.001$), and interaction between assessment date and lure dose ($P=0.002$) were observed. Although one moth was captured in the blank trap of replicate 4 (Fig. 3), this point was not included in the statistical analysis because the moth was captured during a period of peak male flight activity, and its capture was probably a random event. No moths were captured with the 10-μg lure and only a few (*N*=13) with the 100 μg loading.

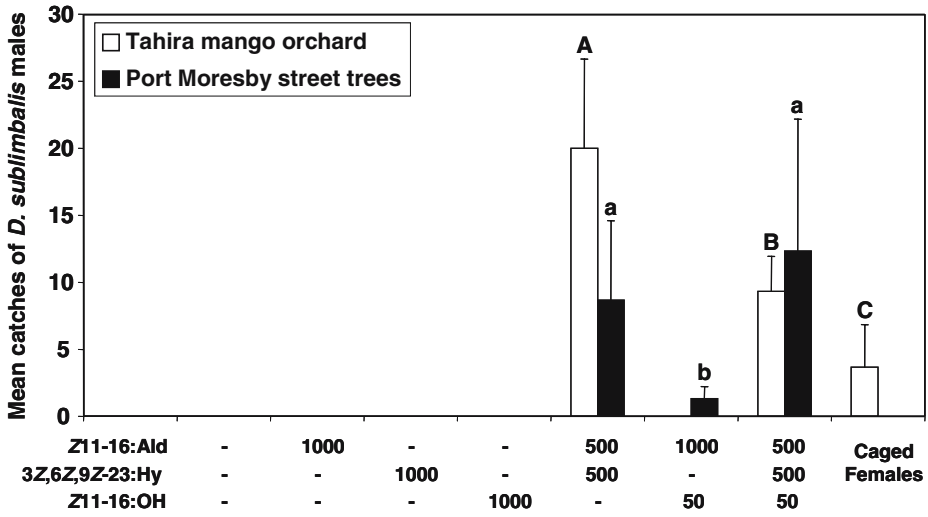


Fig. 2 Mean (+SEM) total catches of *Deanolis sublimbalis* male moths in green delta traps baited with either various combinations of the three compounds that elicited EAD responses or caged female *D. sublimbalis*, in street trees in Port Moresby, and at a mango orchard at Tahira, Central Province, Papua New Guinea (trials A and B). The caged females treatment was not included in the Port Moresby street trees bioassay. Amounts on the *x*-axis are micrograms per lure, and treatments labeled with the same letter are not significantly different ($P>0.05$)

A total of 533 moths were captured in trial C, with similar numbers recorded from traps baited with either the 1000- or 3000-μg lures (235 and 284 male moths, respectively; Fig. 3). Traps baited with the 1000-μg lure and the 3000-μg lure were still capturing male *D. sublimbalis* in similar numbers after 13 weeks. These results suggest that a 1000-μg loading would be an appropriate dose of the binary blend for detection and routine monitoring of male *D. sublimbalis*.

Toward the end of 2004, we carried out a blend trial at the Tahira and PAU sites, using less isomerically pure forms of *Z*11-16:Ald (ca 97% isomerically pure) and 3*Z*,6*Z*,9*Z*-23:Hy (ca 95% isomerically pure), but we only captured moths in traps baited with the caged females (Gibb, unpublished data). These results and the responses of male moths' antennae to *E*11-16:Ald and to isomers of 3*Z*,6*Z*,9*Z*-23:Hy (Fig. 1) suggested the likelihood of an inhibitory effect from one or more of the stereoisomers. Therefore, after obtaining high purity *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy (both >99% isomerically pure), we partially tested this hypothesis in trial D at PAU. A total of 161 moths were trapped in trial D but only with treatment 1, in which the *Z*11-16:Ald was >99% isomerically pure, thus, providing a possible explanation for the initial negative results.

Discussion

We identified *Z*11-16:Ald, and tentatively identified 3*Z*,6*Z*,9*Z*-23:Hy and *Z*11-16:OH, from extracts of the sex pheromone glands of female *D. sublimbalis* moths. None of the

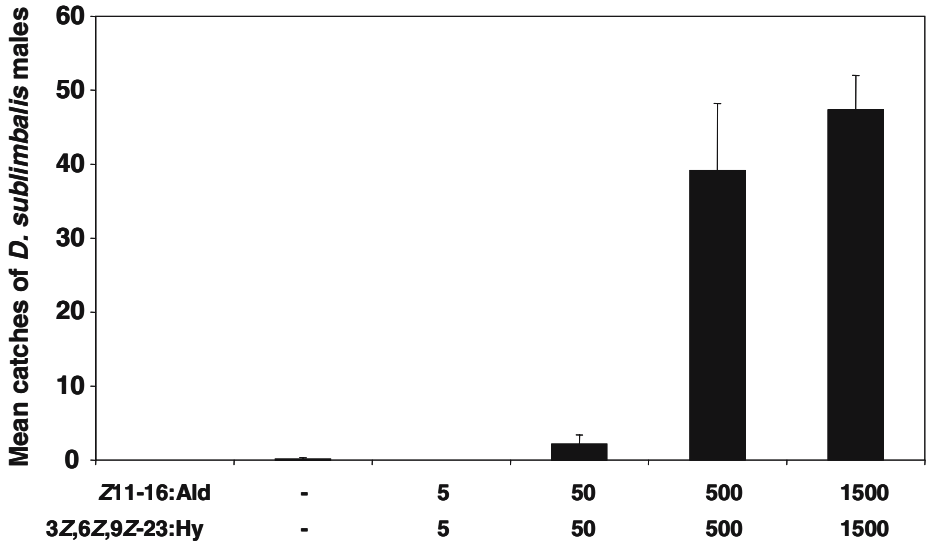


Fig. 3 Mean (+SEM) total catches of *Deanolis sublimbalis* male moths in green delta traps baited with various doses of the two-component blend of (*Z*)-11-hexadecenal and (3*Z*,6*Z*,9*Z*)-tricosatriene, in a mango orchard at Tahira, Central Province, Port Moresby, Papua New Guinea (trial C). Amounts on the *x*-axis are micrograms per lure

individual compounds were attractive and the two-component blend of *Z*11-16:Ald and *Z*11-16:OH was only weakly attractive to male moths. In contrast, the two-component blend of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy was highly attractive, and the ternary blend was no more attractive than the binary blend of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy at the ratios tested. A 1000-μg loading of this binary blend captured ca six times more moths than caged female *D. sublimbalis* (Trial C, Fig. 2). Effective attraction of *D. sublimbalis* male moths required *Z*11-16:Ald of high isomeric purity (trial D), indicating that the (*E*)-isomer is antagonistic. This result suggests that a sympatric species might utilize *E*11-16:Ald as a component of its sex pheromone, presumably in the India–Burma region from where both *M. indica* and *D. sublimbalis* are believed to originate (Waterhouse, 1998).

*Z*11-16:Ald is one of the most frequently identified compounds from lepidopteran sex pheromone glands, with more than 80 species producing it either singly or in combination with other compounds (El-Sayed, 2006). Conversely, the triunsaturated hydrocarbon, 3*Z*,6*Z*,9*Z*-23:Hy, has only been identified twice before in the sex pheromone glands of Lepidoptera, first, as a minor component in two arctiid species (Bell and Meinwald, 1986), and second, as a component in the sex pheromone of a South American crambid (Cabrera et al., 2001). The latter sex pheromone, consisting of *E*11-16:OH and the triene, is similar to the two-component blend identified here from *D. sublimbalis*. These blends of a comparatively short-chain monounsaturated alcohol or aldehyde and a longer-chain polyene were considered to be unusual in lepidopterans (Cabrera et al., 2001; Ando et al., 2004; Jurenka, 2004). However, this type of combination has also recently been reported in three pyralid species (Leal et al., 2005; Millar et al., 2005). Our study is the fifth such example in the superfamily Pyraloidea of a lepidopteran sex pheromone that comprises both a type I and a type II compound, adding additional weight to the suggestion by Millar et al. (2005) that such blends are likely to be relatively common.

The pheromone can now be used for detection and monitoring of this invasive pest in the quarantine area of Cape York Peninsula and the Torres Strait islands. In addition, the pheromone has the potential to be used for control of *D. sublimbalis* in commercial mango plantings using mating disruption or mass trapping (Suckling, 2000). However, trapping systems other than the delta traps used in our trials should be evaluated to avoid trap saturation, as a prerequisite to any mass trapping trials (e.g., bucket traps; see Gibb et al., 2006). Similarly, dispersal and mating behavior of *D. sublimbalis* needs further study before any such direct control attempts. For example, Sujatha and Zaheruddeen (2002) reported that adult *D. sublimbalis* moths are "very sluggish", and therefore, probably not capable of flying long distances, but no empirical data were provided. Limited dispersal would be a distinct advantage for control using mass trapping or lure and kill tactics.

Our data (this study, Trial C) suggest that a 1000-μg loading of the binary mix of *Z*11-16:Ald and 3*Z*,6*Z*,9*Z*-23:Hy on a red rubber septum is an effective attractant for male *D. sublimbalis* moths under tropical field conditions in PNG. However, further development of the pheromone as a management tool will also require its field longevity to be established unequivocally, ideally, in conjunction with a study to compare red rubber septa with alternative lure matrixes.

Acknowledgement We thank Sine Gelua for technical assistance with rearing insects and field bioassays and Adam Matich and Andréa Stephens for commenting on an earlier version of the manuscript. Funding was provided by the Australian Center for International Agriculture Research (ACIAR), Horticulture Australia Ltd (HAL), Growcom (formerly Queensland Fruit and Vegetable Growers Association), and the New Zealand Foundation for Research, Science and Technology (FRST), under "Better Border Biosecurity" and previous programmes, to develop scientific capability to respond to biosecurity incursions.

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J Chem Ecol (2007) 33:591–601
DOI 10.1007/s10886-006-9242-5

Sex Pheromones of Two Melittini Species, *Macroscelesia Japona* and *M. Longipes*: Identification and Field Attraction

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Received: 20 August 2006 / Revised: 30 October 2006 / Accepted: 6 November 2006 /
Published online: 31 January 2007
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Abstract Two Melittini species, *Macroscelesia japona* and *M. longipes* (Lepidoptera: Sesiidae), are native to Japan, but occupy different localities as their host plants seldom grow together. The contents of the sex pheromone gland of adult females of both species, obtained after rearing larvae collected from the field, were investigated by gas chromatograph-electroantennogram detection (GC-EAD) and gas chromatograph-mass spectrometry (GC-MS) analyses. Two GC-EAD-active components were found in a crude extract of *M. japona* female pheromone gland, and identified as (2*E*,13*Z*)-2,13-octadecadien-1-ol (E2,Z13-18:OH) and (2*E*,13*Z*)-2,13-octadecadienal (E2,Z13-18:Ald). The average ratio of these two components was about 1:10. In the field, *M. japona* males were attracted to traps baited with E2,Z13-18:Ald alone, but the strongest attraction was observed with a 1:100 mixture of E2,Z13-18:OH and E2,Z13-18:Ald. The same two components were found in extracts of *M. longipes* females, but in a markedly different ratio. Male *M. longipes* were attracted most strongly to lures containing a 20:1 mixture of

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E2,Z13-18:OH and E2,Z13-18:Ald, although some males were also attracted to lures with E2,Z13-18:OH alone. Although the two species do not generally occur in sympatry, our data indicate that, in the event of overlap, cross attraction of the two species is unlikely.

Keywords (2*E*,13*Z*)-2,13-octadecadien-1-ol · (2*E*,13*Z*)-2,13-octadecadienal · Clearwing moth · Melittini · Reproductive isolation · Lepidopteran pheromones

Introduction

The Sesiidae is a family that includes over 1,000 diurnal species worldwide (Pühringer and Kallies, 2004). Adults of most species have wasp-mimic stripes (yellow or red bands with a black body), in a species-specific pattern that may be involved in mate recognition. In addition to visual cues, sesiids use female sex pheromones in mating (Bûda and Karalius, 1985; Karalius and Bûda, 1993). Since the original study on *Synanthedon pictipes* and *S. exitiosa* by Tumlinson et al. (1974), the sex pheromones of 15 species of Sesiidae have been identified (Ando, 2006). These identifications, however, have been mainly conducted in Europe and the United States, and information about Asian species is limited. About 30 yr ago, sex attractants for two agricultural pests, *S. hector* and *S. tenuis*, were discovered by a field screening test in Japan by using a synthetic pheromone of *S. pictipes* and related compounds (Yaginuma et al., 1976; Tamaki et al., 1977). However, no actual pheromone identifications were published on the Japanese species in this family until our recent study on *Nokona pernix* (Naka et al., 2006).

The Sesiidae are divided into two subfamilies, Sesiinae and Tintiinae, with the former subdivided into six tribes: Synanthedonini, Paranthrenini, Melittini, Sesiini, Osminini, and Cissuvorini. Pheromone studies worldwide have been carried out mainly with species in the tribes Synanthedonini and Paranthrenini. Species in each of the six tribes occur in Asia, but no species in the tribe Osminini are found in Japan (Arita and Ikeda, 2000; Pühringer and Kallies, 2004). The tribe Melittini is one of the largest tribes of Sesiinae, comprising six genera and 150 species in Asia, Africa, and the New World (Pühringer and Kallies, 2004). However, pheromones have been reported only for one species, *M. cucurbitae* (Klun et al., 1990). In Japan, six species in the tribe Melittini, two *Macroscelesia* species and four *Melittia* species, are known (Arita and Ikeda, 2000). Both *Macroscelesia* species are monophagous, feeding on different host plants in different habitats. Larvae of *M. japona* burrow into the stems of *Gynostemma pentaphyllum*, whereas larvae of *M. longipes* bore into the stems of *Actinostemma lobatum* (Arita and Ikeda, 2000). *G. pentaphyllum* grows commonly in Japanese coppices, whereas *A. lobatum* is an endangered species, growing in riverbeds. In Japan, almost all rivers are now enclosed between protective walls, virtually removing all habitats for *A. lobatum*. *M. longipes*, which feeds exclusively on *A. lobatum*, and has shown a consequent reduction in numbers.

Although *G. pentaphyllum* and *A. lobatum* seldom grow together, it is possible that adults of the two moths might coincide as they have similar flight periods and their distributions may be in close proximity. With this in mind, we set about to determine whether the two closely related clearwing moths have different pheromone systems, as well as to increase the information on pheromone components used by Asian sessiid species. This report deals with the identification and field evaluation of the first pheromones from clearwing moths in the tribes Melittini.

Methods and Materials

Insects and Pheromone Extraction About 40 overwintering larvae of *M. japona* were collected in Maihara City, Shiga Prefecture, in November 2003 and vernalized in a refrigerator at 8±1°C without light for 2 mo. After vernalization, insects were kept in a plastic cage at 25±1°C under a 15L: 9D photoperiod until they emerged as adults. Adults were sexed based on their antennae (Arita and Ikeda, 2000). Under laboratory conditions, females exhibited the calling position irregularly during the photophase. Therefore, we observed the behavior of 1- or 2-d-old virgin females and excised their abdominal tips when a calling position was observed. Abdominal tips were placed individually in hexane (250 μl/female) for 5 min to extract the pheromone, before analysis by gas chromatography- electroantennographic detection (GC-EAD; Struble and Arn, 1984) and gas chromatography-mass spectrometry (GC-MS).

For the study on *M. longipes*, about 20 galls containing larvae were collected from a riverbed near Lake Sanaru in Hamamatsu City, Shizuoka Prefecture, in August 2003. The galls were kept at 25±1°C under a 15L-9D photoperiod until the adults emerged after 15–20 days. As *M. longipes* females showed a calling position 1–4 hr after the beginning of the photophase, their abdominal tips were excised 4 hr after the beginning of the photophase. Extracts were made and analyzed as for *M. japona*.

Chromatography and Derivatization of the M. japona Pheromone The crude extract of pheromone glands [10 female equivalents (FE)] was adsorbed on a microcolumn packed with Florisil® (100–200 mesh, Floridin Co., 200 mg). The column was successively eluted with 1 ml each of hexane and 5, 10, 20, and 50% diethyl ether in hexane. Each fraction was concentrated by a gentle nitrogen stream and an aliquot (1 FE) analyzed by GC-EAD. The 5% diethyl ether fraction (9 FE), which included an electroantennogram (EAG)-active aldehyde component, was treated with $NaBH_4$ (0.5 mg) in 2-propanol (0.1 ml) for 3 hr at room temperature. After mixing with water (1 ml), the crude products were extracted with hexane (0.5 ml) and analyzed by GC-MS.

Chemicals (2*E*,13*Z*)-2,13-octadecadienyl acetate (E2,Z13-18:OAc) was supplied by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). The sample included traces (<2%) of some unknown impurities, but not the corresponding alcohol. (2*E*,13*Z*)-2,13-octadecadien-1-ol (E2,Z13-18:OH) was prepared by the hydrolysis of E2,Z13-18:OAc with methanolic NaOH. Other geometric isomers of (2,13)-octadecadien-1-ol were synthesized from 1,9-nonanediol. Their synthesis and structure confirmation by NMR analysis have been reported in a previous paper (Naka et al., 2006). (2*E*,13*Z*)-2,13-octadecadienal (E2,Z13-18:Ald), was obtained by oxidation of E2,Z13-18:OH with pyridinium chlorochromate in CH_2Cl_2. ^{1}H NMR: δ (ppm) 0.90 (t, 3H, *J*=7.3 Hz), 1.2–1.4 (broad s, 16H), 1.50 (m, 2H), 2.02 (m, 4H), 2.33 (dt, 2H, J=7 and 7 Hz), 5.35 (m, 2H), 6.12 (dd, 1H, *J*=7.9 and 15.6 Hz), 6.85 (dt, 1H, *J*=6.8, and 15.6 Hz), 9.50 (d, 1H, *J*=7.9 Hz). ^{13}C NMR: δ (ppm) 14.0, 22.4, 26.9, 27.2, 27.9, 29.2, 29.3, 29.4, 29.5 (× 2), 29.8, 32.0, 32.8, 129.8, 129.9, 133.0, 159.1, 194.2.

Analytical Instruments The GC-EAD apparatus and conditions have been reported (Naka et al., 2006). Briefly, an HP-5890 Series II gas chromatograph, (Hewlett-Packard, Wilmington, DE, USA) with a DB-23 capillary column (0.25 mm×30 m, J&W Scientific, Folsom, CA, USA) was used for the analyses. The oven temperature was maintained at

100°C for 2 min, programmed at a rate of 20°C/min to 175°C, then at 6°C/min to 220°C, and held at this temperature for 15 min. The effluent from the column was split at a ratio of 1:1 into two lines, one of which led to a flame ionization detector (FID) and the other to the EAD. GC-MS analyses were conducted in electron impact ionization mode (EI, 70 eV) using an HP-5973 mass spectrometer interfaced to an HP-6890 gas chromatograph, using the same column and conditions as for the GC-EAD analyses. The ion source temperature was 230°C. ^{1}H and ^{13}C NMR spectra were recorded using a JEOL Alpha 500 Fourier transform spectrometer at 500.2 and 125.7 MHz, respectively, for $CDCl_3$ solutions containing TMS as an internal standard.

Field Tests Attraction of *M. japona* males to synthetic lures was examined at coppices in Ibaraki Prefecture. Test I was conducted in Chikusei and Tsukuba cities from June 30 to July 12, 2003; test II was conducted in Tsukuba City from June 25 to July 10, 2003; and tests III and IV were performed in Tsukuba City from June 17 to July 16, 2004. Test chemicals were absorbed onto a white rubber septum (Sigma-Aldrich Co., St. Louis, MO, USA), which was placed in the center of a sticky board trap (Takeda Chem. Co. Ltd., Tokyo, Japan). Traps were placed 1.5 m above the ground and at least 10 m from each other. Numbers of captured males in each trap were counted every few days. After counting, the traps were rotated to eliminate any positional effect (Latin square design; Perry et al., 1980).

Trials testing *M. longipes* synthetic pheromone were conducted over 4 d in mid-August, 2004 at the riverbed of an oxbow in Fujimi City, Saitama Prefecture. As *M. longipes* is an endangered species, we did not capture males with a sticky trap but recorded their behavior around rubber septa baited with synthetic chemicals. Each septum was fixed to a riverside plant with a safety pin, 50 cm above the ground.

Statistical Analyses The EAG data that used 1 ng of synthetic pheromone compounds (see Fig. 3a and c) were analyzed by one-way ANOVA. Means that were significantly different were separated by Tukey–Kramer's HSD test (Sokal and Rohlf, 1995). For the EAG-dose data, using synthetic E2,Z13-18 compounds and for the field trials, the data were log-transformed before analysis by one-way ANOVA. Means that were significantly different were separated by Tukey–Kramer's HSD test. The level of significance in all tests was set at 5%.

Results

Identification of Pheromone Components of M. japona Male *M. japona* antennae were stimulated by two components, I and II (Fig. 1a). Component I eluted from a Florisil® column with 10% ether in hexane, indicating an alcohol, whereas component II eluted with 5% ether in hexane, indicating an acetate or aldehyde. The mass spectrum of component I (Fig. 1b, c) showed M^+ at m/z 266 (relative intensity: 1.4%), $[M\text{-}H_2O]^+$ at m/z 248 (10.1%), and a series of fragment ions derived from straight-chain compounds, indicating octadecadien-1-ol. The spectrum is similar to that of synthetic E2,Z13-18:OH, which is easily distinguished from spectra of 3,13-octadecadien-1-ol by the relative intensity of M^+ and $[M\text{-}H_2O]^+$ (Naka et al., 2006). The retention time (R_t) of Component I was the same as that of authentic E2,Z13-18:OH, but different from those of the three other geometric isomers [(2*E*,13*E*)-isomer, R_t 11.30 min; (2*Z*,13*E*)-isomer, R_t 11.46 min; (2*Z*,13*Z*)-isomer,

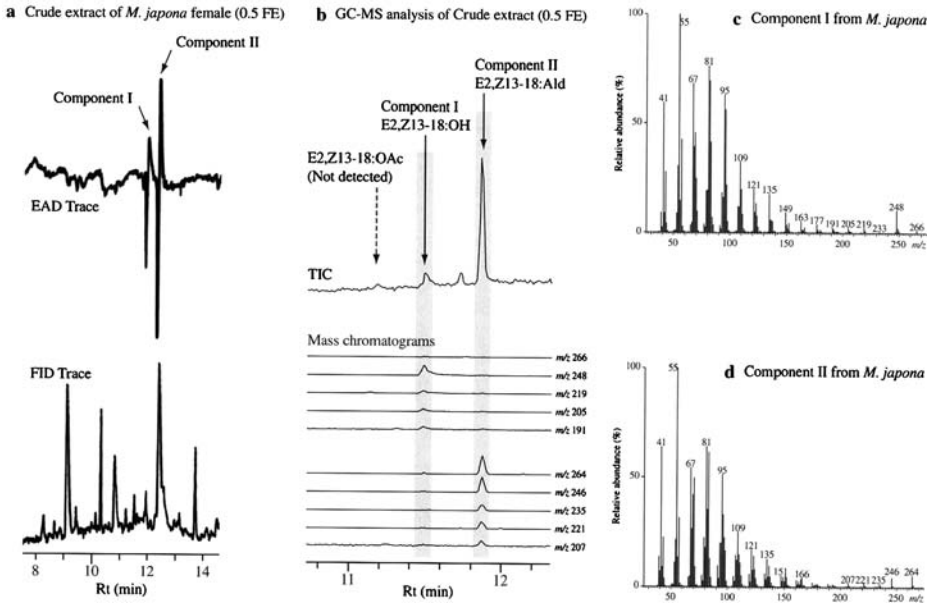


Fig. 1 Analysis of the sex pheromone components of *Macroscelesia* japona by GC-EAD and GC-MS. (a) GC-EAD analysis of pheromone extract (0.5 FE). (b) GC-MS analysis of extract (0.5 FE); total ion chromatogram (TIC) and mass chromatograms monitoring diagnostic ions at *m/z* 266 (M^+ of E2,Z13-18:OH), *m/z* 248 ($[M-18]^+$ of alcohol and $[M-60]^+$ of acetate), 219, 205, and 191 of E2,Z13-18:OH and E2,Z13-18:OAc, and *m/z* 264 (M^+), 246 ($[M-18]^+$), 235, 221, and 207 of E2,Z13-18:Ald. E2,Z13-18:OAc was not detected in the crude extract. (c) Mass spectrum of Component I (E2,Z13-18:OH). (d) Mass spectrum of Component II (E2,Z13-18:Ald)

R_t 11.67 min]. The mass spectrum of component II showed M^+ at m/z 264 (4.4%) and $[M-H_2O]^+$ at m/z 246 (4.4%), indicating an octadecadienal (Fig. 1c, d). This spectrum corresponded closely to that of synthetic E2,Z13-18:Ald. Furthermore, $NaBH_4$ reduction of the 5% ether fraction produced E2,Z13-18:OH, indicating that Component II was E2,Z13-18:Ald. Ten GC-MS injections (1 FE each) of different extracts allowed determination of the mean amounts of the two components: 0.3±1.2 ng/female for E2,Z13-18:OH and 4.2±5.4 ng/female for E2,Z13-18:Ald. The two components were in a ratio of roughly 1:10, although there was considerable variation among samples. No other candidate pheromone chemicals, such as E2,Z13-18:OAc, could be detected (Fig. 1b).

Identification of Pheromone Components of M. longipes Antennae of *M. longipes* males exhibited consistent EAG responses to two compounds with the same retention times as components I and II of *M. japona* (Fig. 2a). GC-MS analysis of the pheromone extract (Fig. 2a–d) confirmed that extract of *M. longipes* pheromone gland contained E2,Z13-18:OH and E2,Z13-18:Ald. Three GC-MS injections (1 FE each) of *M. longipes* extract gave a mean titer of E2,Z13-18:OH of 16.8±8.0 ng/female. E2,Z13-18:Ald was detected in one sample (0.4 ng/female), but not in the other two.

EAG Activities of Synthetic Compounds EAG responses of male antennae to synthetic compounds known to be pheromone components of Sesiidae were determined for the two

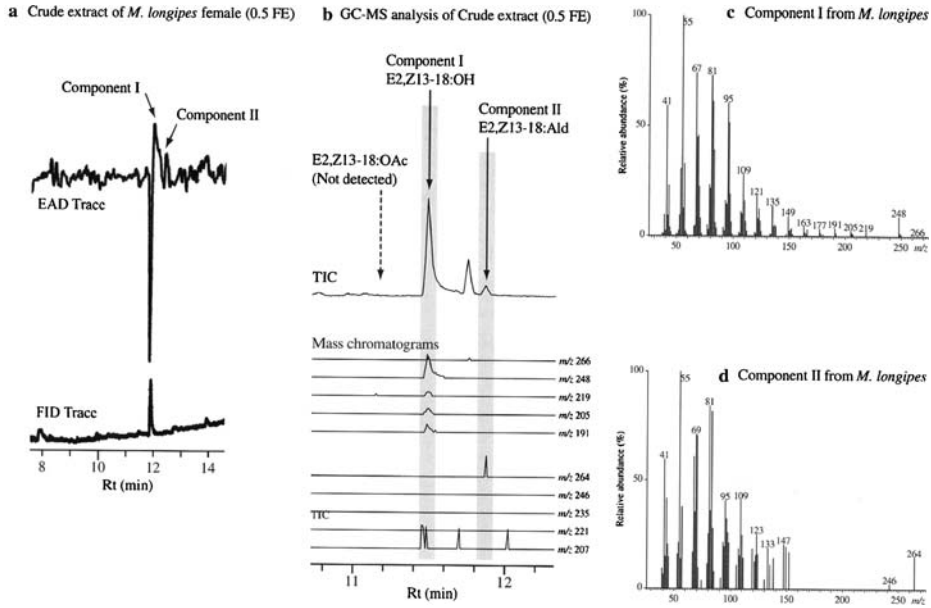


Fig. 2 Analysis of the sex pheromone components of *Macroscelesia* longipes by GC-EAD and GC-MS. (a) GC-EAD analysis of pheromone extract (0.5 FE). (b) GC-MS analysis of extract (0.5 FE); total ion chromatogram (TIC) and mass chromatograms monitoring diagnostic ions at *m/z* 266 (M^+ of E2,Z13-18:OH), *m/z* 248 ($[M-18]^+$ of alcohol and $[M-60]^+$ of acetate), 219, 205, and 191 of E2,Z13-18:OH and E2,Z13-18:OAc, and *m/z* 264 (M^+), 246 ($[M-18]^+$), 235, 221, and 207 of E2,Z13-18:Ald. E2,Z13-18:OAc was not detected in the crude extract. (c) Mass spectrum of Component I (E2,Z13-18:OH). (d) Mass spectrum of Component II (E2,Z13-18:Ald)

Macroscelesia species (Fig. 3). *M. japona* antennae showed the greatest response to E2, Z13-18:Ald, the more abundant component in the pheromone gland of females, and a moderate response to E2,Z13-18:OH, the less abundant compound (Fig. 3a). Responses to both compounds were greater than responses to all other compounds tested (ANOVA, $df=6$, $F=21.50$, $P<0.01$). There were no significant differences in response of antennae with respect to dose of three 2,13-dienyl compounds. However, the relative response to the three different compounds was the same at all doses (E2Z13-18:Ald> E2Z13-18:OH> E2Z13-18:OAc) (Fig. 3b). *M. longipes* antennae showed the greatest EAG response (greater than to all other compounds tested; ANOVA, $df=6$, $F=9.90$, $P<0.01$) to E2,Z13-18:OH, the most abundant compound in the female pheromone gland extract (Fig. 3c). Again, there were no significant differences in response to antennae with respect to dose of the three 2,13-dienyl compounds. However, EAG responses to E2,Z13-18:OH were greater than responses to the other two compounds at all three doses tested (Fig. 3d).

Field Attraction of M. japona Males Of the single-component lures baited with one of the 2,13-dienyl compounds, only traps baited with E2,Z13-18:Ald caught male *M. japona* (Test I, Table 1). When different amounts of E2,Z13-18:OH or E2,Z13-18:OAc were added to E2,Z13-18:Ald, surprisingly, only traps baited with both E2,Z13-18:OAc and E2,Z13-18:Ald caught males (Test II, Table 1). As treatments with E2,Z13-18:OAc appeared to shut down trap capture to all other blends (including to that of E2,Z13-18:Ald alone), further tests were conducted in which the effect of the alcohol component was evaluated in a field

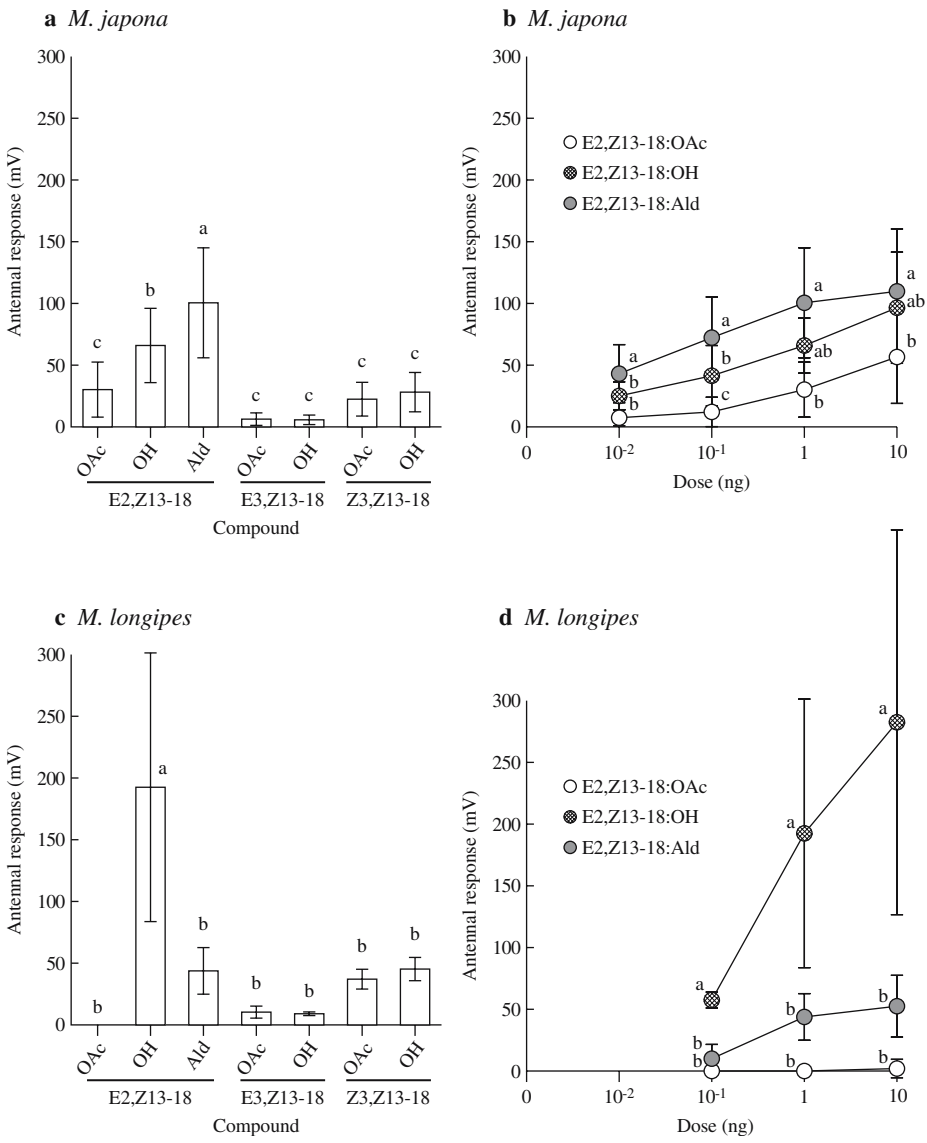


Fig. 3 EAG responses of *Macroscelesia japona* and *M. longipes* males. (a) EAG responses of antennae of *M. japona* males to synthetic standards (1 ng). (b) Responses of antennae of the *M. japona* males to various doses of (2*E*,13*Z*)-2,13-octadecadienyl compounds. (c) EAG responses of antennae of *M. longipes* males to synthetic standards (1 ng). (d) Responses of antennae of the *M. longipes* males to various doses of (2*E*,13*Z*)-2,13-octadecadienyl compounds. Responses are the means of at least ten antennae of *M. japona* and four antennae of *M. longipes*. Values followed by a different letter are significantly different at $P<0.05$ (Tukey–Kramer's HSD test) in (a)–(c). In (d), values followed by a different letter are significantly different at $P<0.05$ (Tukey–Kramer's HSD test) for a given dose of the different compounds

about 4 km from the field testing mixtures of the aldehyde and acetate. Traps baited with lures containing both E2,Z13-18:Ald and E2,Z13-18:OH caught significant numbers of males (Test III, Table 1), with the greatest numbers (significantly greater than for all other treatments) being caught in the 1:100 (E2,Z13-18:OH: E2,Z13-18:Ald) blend. Again binary

Table 1 Attraction of *M. japona* males to lures baited with synthetic (2*E*,13*Z*)-2,13-octadecadienyl compounds[a]

	Content of C18 compounds (μg)			Mean number of catches (Mean ± SD)[a]
	E2,Z13-18:OH	E2,Z13-18:Ald	E2,Z13-18:OAc	
Test I:	0	1,000	0	21.7±4.0
	1,000	0	0	0
	0	0	1,000	0
	0	0	0	0
Test II:	0	1,000	0	0
	100	1,000	0	0
	300	1,000	0	0
	0	1,000	100	15.8±4.1[a]
	0	1,000	300	10.5±6.4[a]
	0	0	0	0
Test III:	0	1,000	0	1.0±1.0b
	100	1,000	0	13.0±7.0[a]
	50	1,000	0	2.3±3.2[b]
	100	1,000	0	0.3±0.6[b]
	300	1,000	0	0
	500	1,000	0	0
	0	0	0	0
Test IV:	0	1,000	0	0.7±1.2b
	0	1,000	10	2.7±3.8[a b]
	0	1,000	50	5.0±3.6[a b]
	0	1,000	100	6.0±4.4[a b]
	0	1,000	300	8.0±3.6[a b]
	0	1,000	500	11.0±2.6[a]
	0	0	0	0

[a] Test I was performed in two fields (N=3); Chikusei City (a private coppice under Kokai-Ohashi Bridge) and Tsukuba City (coppices in NIAES) from June 30 to July 12, 2003. Test II was performed in Tsukuba City (N=4); coppices in NIAES, NIES and Yukari-no-Mori from June 25 to July 10, 2003. Tests III and IV were performed in the same fields as Test II (N=3) from June 17 to July 16, 2004.

[b] Values followed by a different letter are significantly different at the 5% level by Tukey-Kramer test.

blends of E2,Z13-18:Ald and E2,Z13-18:OAc caught significant numbers of male *M. japona* (Test IV, Table 1).

Field Attraction of M. longipes Males Because *M. longipes* males fly slowly, their behavior around synthetic lures could readily be observed in the field. Two behaviors were observed: approaching the lure and touching the lure with forelegs. Males only approached lures containing E2,Z13-18:OH (Table 2). About half of the approaching males touched the lures. The greatest numbers of males that approached lures did so to binary blends of E2,Z13-18:OH and E2,Z13-18:Ald; the numbers that approached the 1,000:50 and 1,000:300 lures were significantly greater than the number that approached the lure containing only E2,Z13-18:OH. Greater numbers of males touched the 1,000:50 lure (E2,Z13-18:OH: E2,Z13-18:Ald) than touched any of the other lures. The numbers of males that approached or touched the binary blend of E2,Z13-18:OH and E2,Z13-18:OAc were not significantly

Table 2 Attraction of *M. longipes* males to lures containing synthetic (2*E*,13*Z*)-2,13-octadecadienyl compounds[a]

Content of C18 compounds (μg)			Mean number of attracted males (Mean ± SD)[a]	
E2,Z13-18:OH	E2,Z13-18:Ald	E2,Z13-18:OAc	Touched	Attracted
1,000	0	0	6.3±1.7[b c]	3.0±1.8[b c]
1,000	50	0	13.8±3.3[a]	9.8±2.2[a]
1,000	100	0	13.0±6.1[a b]	5.3±2.9[b]
1,000	300	0	13.3±2.5[a]	5.5±1.3[b]
1,000	0	100	5.5±2.4[c]	3.0±1.4[b c]
0	1,000	0	0	0
0	0	1,000	0	0
0	0	0	0	0

[a] Test was performed in a riverbed of the Bin-Numa oxbow (Fujimi City, Saitama Prefecture) for 4 d between August 12 and August 23, 2004.

[b] Mean number of 4 days. Values followed by a different letter are significantly different at the 5% level by Tukey–Kramer test.

[c] Attracted: males attracted to within 5 cm. Touched: males touched the lure with their forelegs.

different from the numbers that approached or touched the lure containing only E2,Z13-18:OH (Table 2).

Discussion

GC-EAD, GC-MS analyses of pheromone extracts, combined with field studies, revealed E2,Z13-18:OH and E2,Z13-18:Ald as common pheromone components of two *Macroscelesia* species, *M. japona*, and *M. longipes*. The major component of the former species was aldehyde and that of the latter was alcohol. These compounds were identified by comparison with chemical data of known compounds, not by direct chemical reaction, because of the limited number of available insects. All the dienyl compounds known as Sesiidae pheromones or attractants have a C_{18}-chain skeleton unsaturated at the 3- and 13-positions or the 2- and 13-positions (Ando, 2006). The GC-MS data of the alcohol component of both *Macroscelesia* species strongly indicated a 2,13-dienyl structure (Naka et al., 2006). Configurations of the two double bonds were determined by comparing R_t values of the natural component and the four geometric isomers. The second component was indicated as an aldehyde by its elution time on the DB-23 column. The double-bond positions and configurations of the aldehyde component from *M. japona* females were determined by reduction by $NaBH_4$ to produce the same dienyl alcohol as the other pheromone component. To assist in pheromone identifications of other sessiid moths, we intend to synthesize the four geometric isomers of 2,13-18:Ald.

Compounds with the C_{18} 2,13-dienyl skeleton have been reported as sex pheromones in several species in the lepidopteran families of Sesiidae, Cossidae, and Tineidae. All these compounds have a (2*E*,13*Z*)-configuration; no 2,13-dienes with other configurations have been found in pheromone gland extracts of female moths. (Ando, 2006; El-Sayed, 2006). In the Sesiidae, E2,Z13-18:OH and E2,Z13-18:Ald have been identified only from *Melittia cucurbitae* (Klun et al., 1990) and *Sesia apiformis* (Francke et al., 2004), respectively,

whereas E2,Z13-18:OAc has been identified in a number of species. Interestingly, *Melittia cucurbitae* is taxonomically close to *Macroscelesia* spp.

The reproductive isolation of sibling species, through use of different blends of common sex pheromone components, has been reported in many species of moths (Löfstedt et al., 1991; Ishikawa et al., 1999). Our study revealed that two *Macroscelesia* species produce the same two pheromone components, E2,Z13-18:OH and E2,Z13-18:Ald, but apparently in a different ratio. In accord with this, males of the two species appear to respond optimally to blends similar to those produced by their respective females: 1:100 for *M. japona* males and 20:1 for *M. longipes* males (Tables 1 and 2). Although single-component blends that are attractive to the other species were tested in the respective habitats of the two species, no *M. japona* males approached lures in the habitat of *M. longipes*, and no *M. longipes* males were captured in traps in the test for *M. japona* males. This result suggests that geographical overlap of the species is not common (at least in the areas tested), and precludes any possible hybridization of the two species. However, if there were any geographical overlap, our data suggest that there would be little, if any, cross attraction between the species.

Although E2,Z13-18:OAc was not detected in the pheromone glands of the two *Macroscelesia* species, we were interested in its activity with respect to males of the two species. The acetate appeared to have little effect on *M. longipes* males. However, in the case of male *M. japona*, addition of this compound to E2,Z13-18:Ald gave increased trap captures relative to the latter compound alone. Field Tests II and IV indicated that E2,Z13-18:OAc may have acted as a substitute for E2,Z13-18:OH, the minor pheromone component of *M. japona*. Substitution of a minor component by other analogs has been observed in other lepidopteran species (Linn et al., 1984; Sugie et al., 2003; Inomata et al., 2005). However, in field Test II, the blends of the aldehyde and acetate caught significantly greater numbers of moths than the natural blends of the aldehyde and alcohol, with trap catches to the latter blends being completely suppressed. This preferential response to an unnatural blend over a natural one is perplexing, and suggests that *M. japona* males could be attracted to another species that produces a blend of E2,Z13-18:Ald and E2,Z13-18:OAc. To date, no species using this mixture as a sex pheromone has been identified, and the mixture in our field trials attracted no other species. However, considering the diversity of Sesiidae, and the apparent limited variety of chemicals used by this family as pheromone components, it is possible that some species secrete such a mixture. Further studies on a greater number of species of Sessiidae are necessary to identify such species.

Acknowledgments We are grateful to Mr. R. Wakasugi, D. Wakatsuki, S. Onodera, and students of the Laboratory of Zoology at Meijo University, and Mr. T. Takamura in Nagoya City for collecting galls of *Macroscelesia* spp., Dr. F. Mochizuki of Shin-Etsu Chemical Co., Ltd. for supplying dienyl acetate. We also thank Drs. A. Mochizuki and K. Ito at NIAES, T. Nakazawa at BASE, and Mr. K. Fukuzumi in Nagoya City for advice and information. This work was supported in part by a research fund from Heart Co. Ltd. (Tokyo, Japan).

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J Chem Ecol (2007) 33:603–612
DOI 10.1007/s10886-006-9220-y

Mice Respond Differently to Urine and Its Major Volatile Constituents from Male and Female Ferrets

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Received: 13 July 2006 / Accepted: 3 November 2006 /
Published online: 31 January 2007
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Abstract Our previous chemical investigation showed that the concentrations of urinary volatiles from males were much higher than those from females in the ferret (*Mustela furo*). The current study was designed to examine the behavioral significance and ecological relevance of this difference for one of the main prey of the ferret, the house mouse (*Mus musculus*). Our data showed that male mice displayed no difference in their response to raw male and female ferret urine. However, they showed significantly less response to female mouse urine mixed with ferret urine than to pure female mouse urine, and to female mouse urine mixed with male ferret urine than to female mouse urine mixed with female ferret urine. Furthermore, high levels of the three major volatiles (quinoline, 2,5-dimethylpyrazine, and 4-hepatanone) in male ferret urine were as effective as raw male ferret urine was in inhibiting the response of male mice. We discuss the ecological and behavioral significance of these findings in terms of chemical mimicry and cognitive feature extraction of predator odors in mice.

Keywords Mouse · Ferret · Urine · Odor · Pheromone · Quinoline · 2,5-Dimethylpyrazine · 4-Heptanone · Mimicry · Predator · Prey

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Introduction

Numerous studies have shown that predator odor can modify rodents' behavior and physiology. Further investigation has revealed that some key volatiles from carnivore feces, specialized skin gland secretions, and urine play crucial roles in the process, although they are usually not as effective as the natural scent sources in their entirety (Mason et al., 1994; Müller-Schwarze, 1995; Apfelbach et al., 2005). Few attempts have been made to investigate whether male and female predator' odors can elicit differential responses from their prey (Apfelbach et al., 2005). One exception is the study by Stoddart (1980), although he failed to find any difference in the response of rodents to male and female cat odors. Our recent chemical analysis of ferret (*Mustela furo*) urine revealed that the total amount of volatiles in male urine was 10 times that in female urine. This dramatic difference may provide a source for rodent prey to respond differentially so as to exploit the behavioral difference between male and female ferrets (Zhang et al., 2005).

Although sympatric predators possibly exert a stronger effect than allopatric predators do on their rodent prey (Mappes et al., 1998), the prey's response to the odors of predators is generally considered to be innate due largely to sulfur compounds common in carnivores (Mason et al., 1994). For this reason, search for carnivore scent-based rodent repellents has mainly focused on sulfur-containing compounds (Vernet-Maury et al., 1984; Sullivan et al., 1988a,b; Epple et al., 1995; Burwash et al., 1998; Bramley and Joseph, 2001). However, chemical investigation has revealed that sulfur compounds in secretions or excretions from some carnivores are often minor components or even absent (Preti et al., 1976; Brinck et al., 1983; Schildknecht and Birkner, 1983; Raymer, 1984; Raymer et al., 1985; Service et al., 2001; Buesching et al., 2002a,b). This suggests that nonsulfur compounds may be important in the response of rodents to their predators. Indeed, Nolte et al (1993) reported that several nonsulfur-containing compounds repel mice. One such compound is o-aminoacetophenone, a volatile chemical commonly found in the anal gland secretions of several *Mustela* species (Brinck et al., 1983; Zhang et al., 2003, 2005).

Ferret urinary volatiles are nonsulfur compounds, with quinoline, 2,5-dimethylpyrazine (2,5-DMP), and 4-heptanone as the major constituents (Zhang et al., 2005). The proportions of these three compounds in total urinary volatiles are 39.0%, 25.0%, and 22.4% in males, and 41.9%, 9.2%, and 6.8% in females, respectively (Zhang et al., 2005). Previous work showed that sulfur compounds from the anal glands of ferrets can elicit aversive response from rodents (e.g., Sullivan et al 1988a,b; Mason et al., 1994; Epple et al., 1995). However, some findings suggest that urine (without sulfur compounds) from *Mustela* species, such as American minks (*M. vison*) and ferrets, can also alter the behavior of rodents (Epple et al., 1993; Roberts et al., 2001; Cloe et al., 2004).

Interestingly, both 2,5-DMP and 4-heptanone are also found in urine from female house mice. The former is a female-specific pheromonal compound, whereas the latter is associated with pregnancy and lactation (Novotny et al., 1986; Jemiolo et al., 1987). This coincidence of chemical compounds between predator and prey raises the question of how male mice respond to potentially conflicting information from female conspecifics and their main predator. Our present work, therefore, is aimed at answering the questions of whether ferret male and female urine can elicit differential responses and whether the synthetic analogues of the three major volatile constituents can inhibit the response of house mice. Regarding the first question, because the major urinary volatiles are different between male and female ferrets, we hypothesize that mice can differentiate between male and female ferret urine. For the second question, since the presence of a major predator poses a high risk to mice, the potential attraction of male to female mice could be overcome by the presence of a life-

threatening predator. For this reason, we hypothesize that the responses of mice will be more strongly affected by chemicals unique to predator urine than by chemicals common to the urine of both mice and predator.

Methods and Materials

Experimental Animals

Mice We used 23 naïve male and 12 female ICR/Albino house mice, age 9–10 mo. They were purchased from Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA, and were kept individually in polypropylene cages (27×12×17 cm) in Indiana University Animal Facility. The mice were housed at 21±0.2°C and 50–70% RH. A 12L:12D reverse light cycle regime was used with light on at 2200 hours. Purina Mouse Chow and water were supplied *ad libitum*. The bedding material (sawdust) was changed weekly. Test males used as odor recipients were randomly assigned to two groups for experiment series 1 (12 males) and experiment series 2 (11 males), respectively. The subjects were used in random order. Four estrous females were selected as urine donors.

Ferrets Four adult male and five adult female ferrets in breeding condition (about 1 yr of age) were purchased from Marshall Farms (North Rose, NY, USA). Upon arrival at the Boston University Animal Facility, they were individually housed in modified rabbit cages under a long-day (16L:8D) photoperiod. Ferrets were fed with moistened Purina Ferret Chow once a day, whereas water was available *ad libitum*. The handling procedure for mice and ferrets complied with the institutional guidelines for animal use and care for both Indiana University and Boston University.

Sample Preparation and Experimental Procedure

Quinoline, 2,5-DMP, and 4-heptanone were purchased from Aldrich Chemical Company. They were each diluted to 0.5% in deionized water for the behavioral test. We roughly estimated the concentrations of quinoline, 2,5-DMP, and 4-heptanone to be 0.96, 0.64, and 0.54 ppm, respectively, in male ferret urine by comparing their gas chromatographic peak areas with those of concentration-known internal standards (Zhang et al., 2005). Various odor samples of the synthetic chemicals were created by adding the synthetic compounds to either estrous female mouse urine or female ferret urine, and tested using male mice.

Experiment series 1 To characterize the responses of mice to male and female ferret odor, responses of male mice to a binary choice of raw male or female ferret urine were measured over 4 consecutive days. Next, we tested the responses of male mice to a binary choice of female mouse urine (FMU) with added male or female ferret urine at concentrations of 50%, 20%, and 10%. After finding that all concentrations were effective, we tested the responses of male mice to a binary choice of 10% raw ferret urine (either male or female) in FMU against FMU alone.

Experiment series 2 To investigate the roles of the three major volatile constituents in ferret urine on the responses of male mice to male and female ferret urine odor, we first tested FMU with only one of the synthetic chemicals, at about 40 times its natural level in male

ferret urine, against FMU. Next, we tested the permutations of FMU spiked with one of the three compounds (at about 40 times its level in male ferret urine) against each other in binary choices. Then, we tested all three chemicals (at 40 times their levels in male ferret urine) added to FMU against either FMU or FMU with quinoline (at 40 times the level in male ferret urine). Finally, we tested combinations of FMU contaminated by 10% (v/v) one of the following: (A) spiked female ferret urine (FFU) by the three synthetic chemosignals at 40 times their natural levels in male ferrets; (B) spiked FFU by them at the natural levels in males; (C) 10% raw FFU; and (D) 10% raw male ferret urine.

Urine Collection

Female mouse urine collection We determined female estrus by examining the vaginal smear. Four estrous females, out of 12, were randomly selected and each kept in a metabolic cage for urine collection for 6 hr. Urine was fed to a vial immersed in dry ice. Urine samples were stored at −20°C until used in a behavioral test.

Ferret urine collection As described by Zhang et al. (2005), ferrets were placed individually in a clean cage with a grid floor and a clean collecting pan underneath to collect urine. Fresh urine from each of four males and three females was collected and immediately stored on ice. Urine that was deposited with or next to feces was not collected. All urine samples were stored at −20°C until use. Pooled urine samples of either sex were used.

Behavioral Test

The preference of male mice to two aqueous scent samples was tested in the dark. Immediately before each trial, a test mouse in its home cage was transferred to the test room under dim light. Scent samples were presented to the mouse by a disposable glass capillary (i.d. 1.1–1.2 mm; o.d. 1.3–1.4; 15 cm length; Drummond Scientific Co., Broomall, PA, USA), similar to the methods used by Lai et al. (1996) and Zhang et al. (2001). Each capillary contained a 10-μl sample aliquot. The opening of the capillary was sealed by Tube Sealing Compound N:O 510 (Chase Instruments Corp., Rockwood, TN, USA) so as to suspend the aqueous sample inside the capillary 1 cm from the sample-containing end. The sample-containing end of the capillary was presented to a mouse, whereas the other end was held by an observer wearing disposable plastic gloves. Two aqueous samples were presented simultaneously to the test subject for 3 min after it first showed a sniffing response. The two capillaries were lowered through the wire lid and kept approximately 2 cm apart. The time that the mouse spent sniffing within 1 cm from the tip and licking the end of the capillary was recorded for both treatments. A mouse was used only once a day. Subjects whose investigating time was less than 1 s on a day were excluded for that day.

Statistical Analysis

Wilcoxon matched-pairs signed-rank test was used for behavioral response using the program SPSS (Version 10.0). The level of significance was set at $\alpha=0.05$ for all tests.

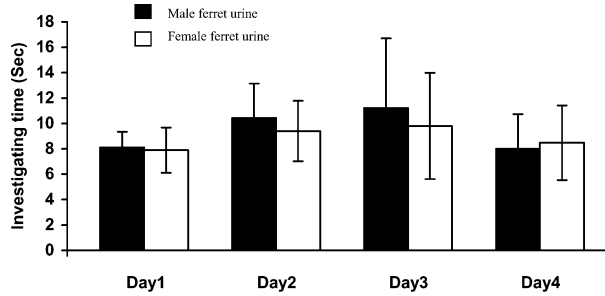


Fig. 1 Responses (mean ± SE) of male mice to male (black) and female (white) ferret urine over four consecutive days

Results

Experiment Series 1. Effects of Male and Female Ferret Urine on Responses of Male Mice

Male mice did not show a differential response to male and female ferret urine in the 3-min daily test on each of 4 successive days (Day 1: N=12, Z=0.079, P=0.938; day 2: N=12, Z=1.177, P=0.239; day 3: N=12, Z=1.098, P=0.272; day 4: N=12, Z=0.314, P=0.754) (Fig. 1). However, when 50%, 20%, or 10% of ferret urine was added to FMU, male mice spent less time investigating FMU mixed with male ferret urine than that mixed with female ferret urine (N=12, Z=2.824, P=0.005; N=11, Z=2.667, P=0.008; N=11, Z=2.756, P=0.005) (Fig. 2). Compared with FMU, adding either 10% male or female ferret urine to FMU reduced the responses of male mice (N=12, Z=3.060, P=0.002 and N=11, Z=2.667, P=0.008, respectively, Fig. 2).

Experiment Series 2: Roles of Three Major Constituents in Eliciting Responses of Mice

Effects of Single Major Components

Male mice reduced investigating time to FMU with quinoline (N=12, Z=2.667, P=0.008), 2,5-DMP (N=10, Z=2.497, P=0.013), or 4-heptanone (N=11, Z=2.134, P=0.033) compared with pure FMU (Fig. 2). Furthermore, male mice spent less time investigating FMU with quinoline compared with FMU with either 2,5-DMP (N=12, Z=2.667, P=0.008) or 4-heptanone (N=10, Z=2.293, P=0.022). They did not respond differently between FMU containing 2,5-DMP and that containing 4-heptanone (N=10, Z=0.968, P=0.330) (Fig. 3).

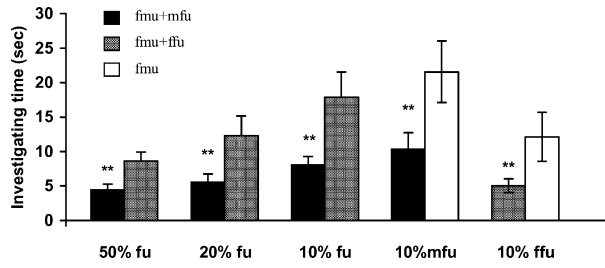


Fig. 2 Responses (mean ± SE) of male mice comparing binary choices between female mouse urine (fmu) mixed with various concentrations of ferret urine (fu), either male (mfu) or female (ffu). Additionally, binary choices between fmu and fmu plus 10% mfu or 10% ffu were compared. **P<0.01; *P<0.05

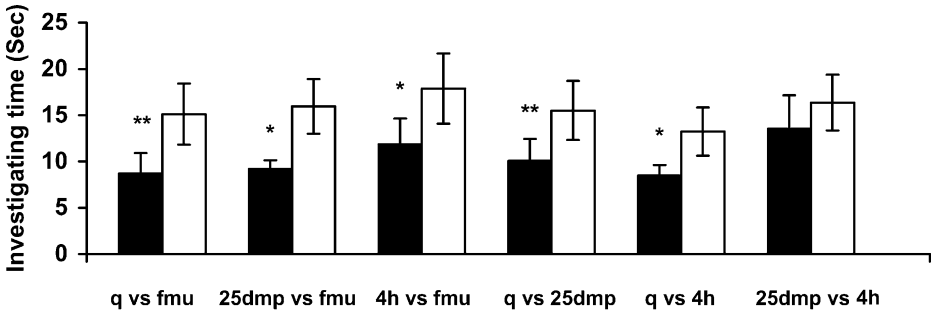


Fig. 3 Responses (mean ± SE) of male mice to female mouse urine (fmu) spiked with quinoline (q), 2,5-dimethylpyrazine (25dmp), or 4-heptanone (4h) in various binary choices. $^{**}P<0.01$; $^{*}P<0.05$

Combined Effect of the Three Major Components

FMU with quinoline, 2,5-DMP, and 4-heptanone together, reduced the investigative response of male mice compared to pure FMU ($N=10$, $Z=2.803$, $P=0.005$) or FMU with quinoline ($N=12$, $Z=2.432$, $P=0.015$) (Fig. 4).

Effect of Mimicking Male Ferret Urine by Spiking Female Ferret Urine with the Three Compounds

Male mice showed less response to FMU scented by 10% female ferret urine containing the three synthetic compounds at about 40 times their natural levels in male ferret urine, than to the same treatment with the three synthetic compounds at roughly the natural levels of male ferrets ($N=10$, $Z=2.089$, $P=0.037$). The response of mice to FMU mixed with 10% female ferret urine, with the three compounds at roughly their natural levels in male ferret urine, was also reduced compared to their response to FMU mixed with 10% raw female ferret urine ($N=10$, $Z=2.497$, $P=0.013$). There was no difference in response to FMU plus the

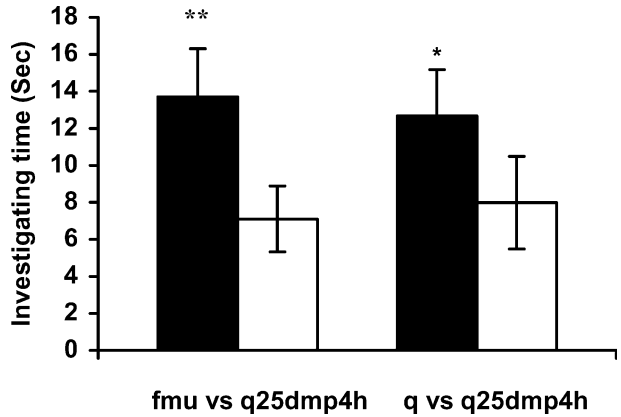


Fig. 4 Responses (mean ± SE) of male mice to female mouse urine (fmu) spiked with quinoline (q), 2,5-dimethylpyrazine (25dmp), and 4-heptanone (4h) together, versus either fmu alone or fmu with *q* only. $^{**}P<0.01$; $^{*}P<0.05$

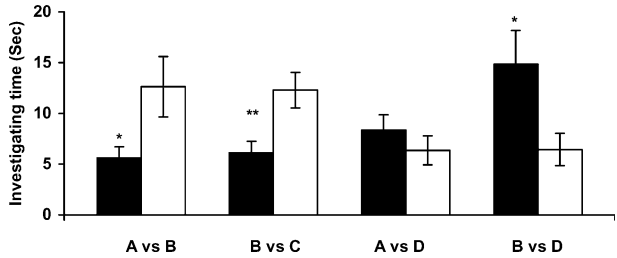


Fig. 5 Binary choices testing the responses (mean ± SE) of male mice to female mouse urine (fmu), mixed with 10% of the following: A= female ferret urine (ffu) plus quinoline (q), 2,5-dimethylpyrazine (25dmp), and 4-hepatanone (4h), all at concentrations 40 times that found in natural male ferret urine (mfu); B= ffu plus *q*, 25 dmp, and 4h, all at natural concentrations found in male ferret urine (mfu); C= ffu; D= mfu. $^{**}P<0.01$; $^{*}P<0.05$

treated female ferret urine with three synthetic compounds, at about 40 times their natural levels in male ferret urine, compared to FMU plus 10% male ferret urine ($N=10$, $Z=1.274$, $P=0.226$). In contrast, mice showed a greater response to FMU plus the three synthetic compounds, at about their natural levels in male ferret urine, compared to FMU plus 10% male ferret urine ($N=10$, $Z=2.089$, $P=0.037$) (Fig. 5).

Discussion

In experiment series 1, we found that mice did not show a difference in response to pure male and female predator odor, consistent with previous work (Stoddart, 1980). However, when female mouse urine was used as a background odor, male mice showed a differential response between sexually dimorphic male and female ferret urine (Zhang et al., 2005). Hence, our hypothesis that mice are able to discriminate between male and female ferret urinary odors is supported. A reason for the apparent discrepancy between the results may be that the response of mice to ferret odor is context-dependent. Urine of male and female ferrets by itself might represent an equal level of threat to mice. However, when mixed with female mice urine, a situation was created for male mice to leverage between a potential benefit (female mouse as a mate) and a cost (the presence of a ferret), and male mice showed behavioral differentiation between the male and female ferret urine.

In experiment series 2, our results suggest that adding any of the synthetic versions of the three compounds to the urine of estrous female mice reduced investigation by male mice. In particular, quinoline, present in ferrets but absent in mice, exhibited a stronger inhibitory effect than the other two chemicals (which are present in both ferrets and female mice) (Andreolini et al., 1987; Jemiolo et al., 1987; Zhang et al., 2005). Thus, our second hypothesis is also supported. Researchers have shown that titers of 2,5-DMP and 4-heptanone in female mice are related to the nonreceptive condition (Novotny et al., 1986; Andreolini et al., 1987; Jemiolo et al., 1987). The compound 2,5-DMP is a mouse pheromone component, which is excreted at a high level by crowded female mice, with the vital behavioral function of the Lee-Boot effect. Its level goes up during diestrous days and down on the estrous day (Novotny et al., 1986; Andreolini et al., 1987; Jemiolo and Novotny, 1993). 4-Heptanone is found in the urine of female mice, and attains a high level in pregnant and lactating females (Jemiolo et al., 1987). That is, these compounds exist in ferrets [both males and females, albeit in different quantities, see Zhang et al. (2005)] and also characterize nonreceptivity of

female mice. This may be the reason why their increases in the urine of estrous females reduced the investigative response of males. Furthermore, the coincidence in the timing of the increase of 2,5-DMP and 4-Heptanone in the urine of female mice and when male mice are unwanted supports the contention that females might chemically deceive males by mimicking the presence of ferrets. This deception could cause an opposing selection pressure for males to evolve an anti-deceptive response. Indeed, our study shows that males exhibited a stronger response to quinoline, which is present in ferrets but not female mice, than to either 2,5-DMP or 4-heptanone. In other words, quinoline may convey unambiguous information about the presence of ferrets. This presumed chemical mimicry, in terms of the function served for the mimicker, would be fundamentally different from other known mimicry systems (e.g., Pasteur, 1982; Dettner and Liepert, 1994). Further studies are needed to substantiate this as well as to investigate how such a system might evolve.

Of the three major volatile compounds in ferret urine, quinoline had the strongest deterring effect. This result demonstrates an evolutionary convergence in the response of a variety of prey animals because quinoline is also an effective repellent for ants, spiders, cockroaches, and frogs (Eisner et al., 1997). Interestingly, apart from ferret urine, quinoline is found mainly in the thoracic glands of phasmid insects (*Oreophoetes peruana*) as a defensive substance (Schildnecht et al., 1986; Zhang et al., 2005).

Our study supports the general rule that for prey recognition, individual components of an odor are less effective than the whole (Müller-Schwarze, 1995; Mason et al., 1994; Schulte et al., 1994; Apfelbach et al., 2005). This is shown by the combination of the three components being more effective than the most effective single compound (quinoline), and by the three compounds together, at their natural concentrations, being less effective than male ferret urine. With regard to the latter point, the three component blend was only as effective as male ferret urine when presented at levels much higher than their natural levels. This suggests that other minor constituents in ferret urine may also play a role in eliciting the aversive response. One possible compound is *o*-aminoacetophenone, which is a repellent for birds and mice (Nolte et al., 1993).

Acknowledgments We are grateful to Dr. Janne Sundell for valuable comments on our earlier draft. We are grateful to Drs. Sarah K. Woodley and Michael J. Baum who provided us with ferret urine. This research was supported by grants [No. DC 24180 to M.V. N] from the National Institute of Deafness and Other Communication Disorders, National Institute of Health (NIH), and US Department of Health and Human Services. The manuscript preparation and data processing were partially supported by the CAS Innovative Research International Partnership Project (CXTDS2005-4).

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J Chem Ecol (2007) 33:613–630
DOI 10.1007/s10886-006-9247-0

Characterization and Comparison of Major Urinary Proteins from the House Mouse, *Mus musculus domesticus*, and the Aboriginal Mouse, *Mus macedonicus*

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Received: 15 September 2006 / Accepted: 18 December 2006 /
Published online: 1 February 2007
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Abstract Urine from the house mouse, *Mus musculus domesticus*, contains a high concentration of major urinary proteins (MUPs), which convey olfactory information between conspecifics. In wild populations, each individual expresses a different pattern of around 8 to 14 electrophoretically separable MUP isoforms. To examine whether other *Mus* species express MUPs and exhibit a similar level of individual heterogeneity, we characterized urinary proteins in urine samples from an aboriginal species, *Mus macedonicus*, captured from different sites in Turkey. Anion exchange chromatography and electrospray ionization mass spectrometry demonstrated that *M. macedonicus* urine contained a single major peak of mass 18,742 Da, and in contrast to *M. m. domesticus*, all individuals were the same. The *M. macedonicus* masses were not predicted from any known MUP gene sequence. Endoproteinase Lys-C (Lys-C) digestion of the purified *M. macedonicus* urinary protein followed by matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry demonstrated that it shared considerable, but not complete, sequence homogeneity with *M. m. domesticus* MUPs. Three *M. macedonicus* Lys-C peptides differed in mass from their *M. m. domesticus* counterparts. These three peptides were further characterized by tandem mass spectrometry. The complete sequences

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of two were determined, and in conjunction with methyl esterification, the amino acid composition of the third was inferred, and the sequence narrowed down to three permutations. The complete *M. macedonicus* sequence contained a maximum of seven amino acid substitutions, discernible by tandem mass spectrometry, relative to a reference *M. m. domesticus* sequence. Six of these were on the surface of the molecule. Molecular modeling of the *M. macedonicus* sequence demonstrated that the amino acid substitutions had little effect on the tertiary structure. The differences in the level of heterogeneity between the two species are discussed in relation to their environment and behavior. In addition, the differences in protein structure allow speculation into molecular mechanisms of MUP function.

Keywords Aboriginal mouse · *De novo* protein sequencing · House mouse · Major urinary proteins · Peptide mass spectrometry · Protein mass spectrometry · Protein sequence heterogeneity

Introduction

House mice, *Mus musculus domesticus*, have evolved intricate and sophisticated methods of chemical communication (Hurst, 1987; Hurst and Beynon, 2004). The major urinary proteins (MUPs) are key components of this signaling system (Beynon and Hurst, 2004). MUPs are eight stranded beta-barrel pheromone binding proteins (Adams and Sawyer, 1990; Flower et al., 1993), encoded by a multigene family, expressed in the liver, and secreted into the urine via the kidneys (Finlayson et al., 1965). A number of small, volatile molecules have been identified as endogenous MUP ligands, most of which are reproductive priming pheromones (Bacchini et al., 1992; Robertson et al., 1993; Novotny et al., 1999) that also influence behavior (Malone et al., 2001). MUPs prolong the release of the volatile ligands from scent marks and may also protect them from oxidation (Hurst et al., 1998). However, this does not explain the extreme polymorphism that is a feature of these proteins in *M. m. domesticus*. Each individual expresses around 8 to 14 electrophoretically separable MUP isoforms, with only very closely related individuals (Hurst et al., 2001) or inbred laboratory mice (Robertson et al., 1996) expressing the same pattern. We have demonstrated that this extreme polymorphism provides a genetically stable method of communicating the individual ownership of scent marks (Hurst et al., 2001, 2005).

Knowledge of the structure and function of MUPs has mostly been derived from *M. m. domesticus*. These mice are thought to have become commensal some 10,000 yrs ago in the Fertile Crescent, utilizing some of the earliest human settlements there (Cucchi et al., 2005). Therefore, the history of *M. m. domesticus* has become inextricably intertwined with that of humans. Their innate agility and flexibility has proved ideal for the continued exploitation of human populations as a ready source of food and other resources, which has facilitated their spread from the Fertile Crescent to all parts of the world (Silver, 1995). Where food resources are abundant (for example around grain stores and livestock housing), population densities typically reach high levels (Berry, 1981; Bronson, 1979), and mice live in territorial social groups in which the ranges of many individuals overlap (Hurst, 1987; Barnard et al., 1991). *Mus m. domesticus* is one of at least three subspecies of house mice that have parapatric distributions and that interbreed where they make contact (Boursot et al., 1993). There are also three other *Mus* species (*Mus macedonicus*, *Mus spretus*, and *Mus spicilegus*) that are closely related to and occur sympatrically with *M. musculus* in Europe

and the Middle East (Boursot et al., 1993; Suzuki et al., 2004). These species are termed aboriginal mice as, unlike the commensal *M. musculus*, they live independently of humans. Commensal mouse populations differ in a number of aspects of their behavior compared to free living aboriginal species (Patris and Baudoin, 1998; Ivantcheva and Cassaing, 1999). Here, we studied the aboriginal mouse, *M. macedonicus*, a short-tailed species that is found in Greece, Turkey, and elsewhere in the Middle East. The aim of this study was to characterize MUPs from *M. macedonicus* and compare them to the well-characterized MUPs from *M. m. domesticus*.

Methods and Materials

Animals and Sampling

Adult male *M. macedonicus* and *M. m. domesticus* urine donors were caught during May–July 2001 in Sherman traps from four locations dispersed around Turkey (Fig. 1). Urine voided by the animals in response to handling (typically 20–50 μl) was collected into 1.5 ml capped polypropylene test tubes and frozen within 5 min. All samples were subsequently stored at −20°C before analysis. Reference MUPs were obtained from pooled urine samples from adult male C57BL/6 mice. These were obtained by bladder massage, frozen immediately, and stored at −20°C.

Urine Sample Preparation

Urine samples were desalted before further analysis with Vivaspin 5,000 Da molecular weight cutoff centrifugal concentrators (Sartorius, Epsom, UK). Typically, a 50 μl urine sample was placed in a prerinsed concentrator and made up to a final volume of 500 μl with distilled water. The sample was then centrifuged at 10,500×*g* for 15 min, which was sufficient to reduce it to 50 μl. This was then expanded to 500 μl with distilled water, and the

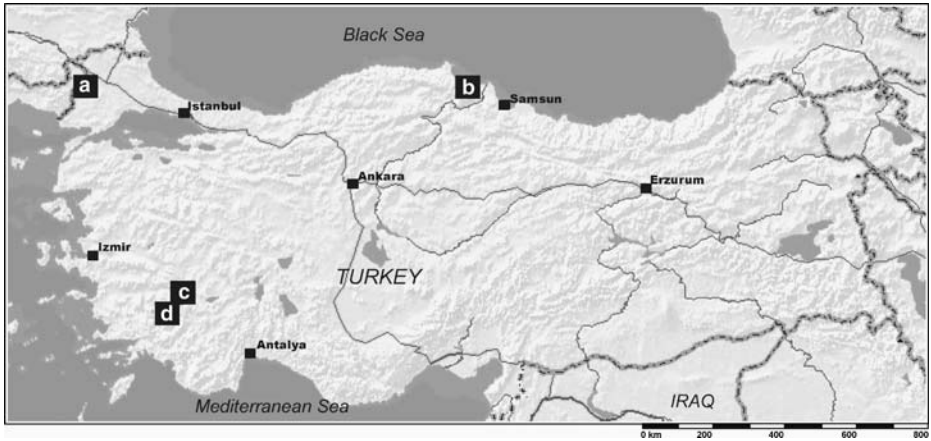


Fig. 1 Map of *Mus macedonicus and M. musculus domesticus* collection sites. Wild specimens of *M. macedonicus* and *M. m. domesticus* were collected for urine samples from four locations in Turkey, labeled (a–d). *Mus macedonicus* were caught in sites: (a), Eskiköy, Uzunkoprü, Edirne, 41°20′ N 26°37′ E (sample 123) and (c), Korucuk, Denizli, 37°49′ N 29°09′ E (samples 129 and 131), whereas *M. m. domesticus* were from (b), Kozköy, Yakakent, Samsun, 41°38′ N 35°31′ E (sample 171) and (d), Denizli, 37°47′ N 28°59′ E (samples 141 and 145)

process was repeated. The sample was then removed from the apparatus and stored at −20°C until required. An identical process was used to desalt fractions collected from anion exchange chromatography before analysis by electrospray ionization mass spectrometry (ESI-MS)

Ion Exchange Chromatography

Anion exchange chromatography was performed on a Dionex Bio-LC platform fitted with a Dionex ProPac SAX column (2×250 mm) and a ProPac SAX guard column (2×50 mm). In all cases, the column flow rate was 0.2 ml/min. After equilibration with 20 mM Tris, pH 8.5, desalted urine samples, (typically 1–5 μl) were loaded onto the column. Samples were eluted from the column with a linear NaCl gradient of 0–500 mM in 30 min. The eluent from the column was monitored at 214 nm in a flow cell of 9 mm path length. Where applicable, fractions were collected by hand directly after passage through the flow cell. All aspects of data acquisition and processing were controlled through the Dionex Chromeleon software.

Proteolysis of MUPs

Anion exchange purified MUPs were proteolyzed by endoproteinase Lys-C (Lys-C), endoproteinase Glu-C (Glu-C), or trypsin (Roche Diagnostics, Lewes, UK). An aliquot (typically 100 μl) of the MUP fraction was reduced with 10 mM 2-mercaptoethanol for 1 hr at room temperature (RT) or with 10 mM dithiothreitol (DTT) at 55°C for 1 hr. In some instances, cysteine residues were carbamidomethylated by incubating the reduced protein preparation with a 55-mM final concentration of iodoacetamide for 1 hr at RT. Removal of reduction and carbamylation reagents was achieved by protein precipitation or centrifugal filtration in a Vivaspin apparatus as described previously. Protein precipitation was achieved by the addition of an equal volume of 20% (w/v) tricarboxylic acid (TCA). After centrifugation at 14,000×*g* for 10 min, the supernatant was discarded, and the precipitate was washed twice with diethyl ether. Residual ether was removed from the sample by incubation at 40°C for 10 min, and the precipitate was resuspended in its starting volume of either 100 mM Tris, pH 8.5 (Lys-C and Glu-C) or 100 mM Tris/2 mM $CaCl_2$, pH 8.5 (trypsin). Protease solution (2 μl of 0.1 mg/ml) was added to the resuspended precipitate, which was then incubated overnight at 37°C. The reaction was subsequently stopped by addition of a 5-μl aliquot of formic acid. Samples prepared by Vivaspin desalting were digested under identical conditions.

Esterification of MUP Lys-C Peptides

Esterification of carboxyl groups within MUP Lys-C peptides was achieved according to Shevchenko et al. (2003). Lys-C peptides from digests of *M. macedonicus* and *M. m. domesticus* MUPs were initially desalted into a solution of 0.1% (v/v) trifluoro-acetic acid (TFA)/50% (v/v) acetonitrile with Zip-Tips (Millipore, Billerica, MA, USA). This solution was subsequently reduced to dryness in a vacuum centrifuge. A 1 ml aliquot of ethanol was placed in a 1.5-ml test tube and placed at −20°C for 15 min, after which, a 150 μl aliquot of acetyl chloride was added, and the mixture was incubated at room temperature for 10 min. The dried peptides were immediately treated with 15 μl of this mixture and incubated at room temperature for 45 min before drying in a vacuum centrifuge. The dried, esterified peptides were finally dissolved in MALDI matrix solution (see below) before matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry

MALDI-TOF mass spectrometry of MUP digests was performed on a Waters-Micromass M@LDI instrument. Peptide mixtures from proteolytic digests of MUPs were mixed in a 1:1 ratio with a matrix solution consisting of saturated α-cyano-4-hydroxy-cinnaminic acid (Sigma Chemical, Poole, UK) in 50% (v/v) acetonitrile/0.2% (v/v) TFA. A 1 μl aliquot of this preparation was deposited on the MALDI target and allowed to dry at RT. Spectra were subsequently acquired between 1,000 and 4,000 Th with the laser energy optimized to give the best signal to noise ratio for each sample. The laser firing rate was 5 Hz and 10 spectra (collected over 2 sec) were combined. The final mass spectrum was a combination of 10–15 such combined data sets, representing 100–150 individual laser shots. All aspects of data acquisition, processing, and machine management were controlled through the MassLynx software suite (versions 3.5 and 4.0).

Electrospray Ionization (ESI) Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MSMS) were performed on a Micromass Q-ToF Micro instrument, fitted with a nanospray source. The electrospray was created from a silver-coated glass capillary with a 10-μm orifice (New Objective, Woburn, MA, USA), held at a potential of +2,000 V relative to the sample cone. For measurement of the mass of intact MUPs, a desalted sample was introduced into the mass spectrometer by syringe pump infusion (Harvard Instruments Ltd, Edenbridge, UK) at a rate of 0.5 μl/min. In this case, the instrument was operated in TOF only mode, with the quadrupole analyzer operating in R_f only mode to allow transmission of all ions. Raw data were gathered between 700 and 1,400 Th at a scan/interscan speed of 2.4/0.1 sec. These raw data were subsequently de-convoluted using the MaxEnt 1 module contained within the MassLynx 3.5 software. To create the MaxEnt damage model, peak width and resolution parameters of 0.75 and 1 Da/channel were used, respectively, and data were processed over the mass range 18,400–19,000 Da.

De novo Sequencing

For tandem mass spectrometry on peptides derived from MUP proteolytic digests, samples were introduced into the mass spectrometer at 0.2 μl/min following reversed phase high pressure liquid chromatography (RP-HPLC) (see below). In this case, the initial quadrupole analyser was set to allow passage only of selected precursor ions to a gas cell, where they were fragmented by collision with argon. The mass of the resulting fragment ions was then measured by the TOF analyzer. Selection of precursors and fragmentation energy were controlled automatically by using the data dependent acquisition facility within the MassLynx software. Precursor spectra were acquired between m/z 400 and 1,500 at a scan/interscan speed of 2.4/0.1 sec. Product ion spectra were acquired between m/z 100 and 2,000 at a scan/interscan speed of 1.0/0.1 sec. Raw product ion spectra were de-convoluted using the MaxEnt 3 algorithm within the MassLynx software. The charge state of the parent peptide was determined from the isotope envelope in the precursor spectrum. Interpretation of product ion spectra and the determination of peptide sequences were facilitated by the PepSeq module within MassLynx.

Reverse Phase-HPLC

Before ESI-MSMS, MUP peptides were separated by RP-HPLC on a Dionex Ultimate system. The system was fitted with a PepMap C18 column (LC Packings, Camberley, UK), 15 cm×75 μm, bead size 3 μm and pore size 100 Å. Before separation, aliquots (2–5 μl) of MUP peptides were desalted in-line using a Dionex Switchos apparatus, fitted with a 1 mm×300 μm, C18 precolumn. The precolumn was initially equilibrated in 0.2% (v/v) formic acid at 30 μl/min. Peptides were then loaded and washed for 3 min at the same flow rate and then eluted with 90% acetonitrile/0.2% formic acid, introduced as a linear gradient of 0–50% in 30 min at 0.2 μl/min. The column eluent was monitored by UV absorbance at 214 nm, before delivery to the mass spectrometer.

Molecular Modeling

Protein sequences derived from the *M. macedonicus* MUPs were used to direct homology models. Briefly, protein structures were modeled in the structure of MUP1 1I04.PDB (Timm et al., 2001). After alignment of the two sequences (no insertions or deletions were necessary), the models were built to the highest quality possible using the Modeler package of the Discovery Studio package (Accelrys, Cambridge, UK, ver 1.5). Because the four C-terminal most residues of MUP1 cannot be defined because of main chain flexibility (Krizova et al., 2004), these residues were not modeled.

Results and Discussion

We analyzed urine from three samples each of *M. macedonicus* and *M. m. domesticus*, obtained from mice caught from geographically distinct locations (Fig. 1). The two species demonstrated different patterns of MUP expression. When the urinary proteins were resolved by ion exchange chromatography, the elution profile of *M. macedonicus* urine consisted of a single major peak and two barely detectable smaller peaks (Fig. 2a). The retention time of the major peak expressed by each individual was similar, (maximum absorbance at 21.69, 21.83, and 21.86 min), suggesting little difference in their MUP profiles. By contrast, the *M. m. domesticus* samples were noticeably more complex and varied among individuals in the number of major and minor peaks (Fig. 2b). Furthermore, the retention times of the major peaks differed by more than 0.25 min among samples, contrasting strongly with the invariable elution times of the major peak in the *M. macedonicus* samples. It is probable that such differences in elution time reflect different proteins (Robertson et al., 1996). Each of the three *M. m. domesticus* samples also differed in the relative proportions of the peaks, which added to the overall level of heterogeneity. For *M. m. domesticus*, such heterogeneity is not surprising and is similar to that observed previously in wild *M. m. domesticus* populations (Robertson et al., 1997; Pes et al., 1999; Veggerby et al., 2002; Beynon and Hurst, 2004). Of greater interest was the relative simplicity and the lack of comparable heterogeneity in the *M. macedonicus* urine samples, which we have never observed in *M. m. domesticus* samples caught from many different locations.

We previously demonstrated the utility of mass spectrometric analysis of intact urinary MUPs (Evershed et al., 1993; Robertson et al., 1996, 1997; Beynon et al., 2002). All six samples generated complex and overlapping mass spectra corresponding to multiple charged protein variants that were readily transformed into true mass spectra (Fig. 3). The

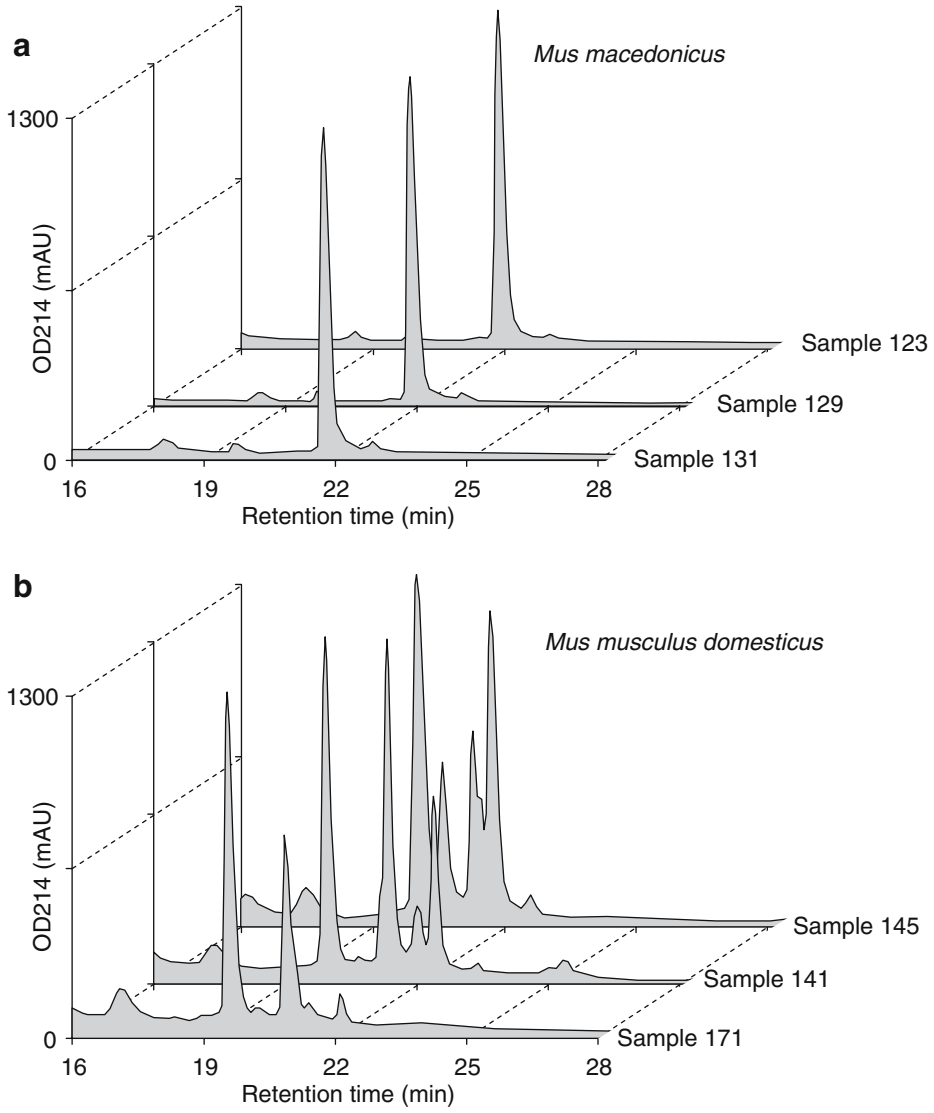


Fig. 2 Anion exchange chromatography profiles of MUPs from three urine samples of (a) *Mus macedonicus* (123, 129, and 131) and (b) *M. m. domesticus* (141, 145, and 171). MUPs were desalted with a centrifugal concentrator (see Methods and Materials section for details). Anion exchange chromatography was performed on a 2×250 mm Propac SAX anion exchange column mounted on a Bio LC chromatography platform (Dionex, Camberly, UK). The column was equilibrated with 20 mM Tris buffer, pH 8.5 at 0.2 ml/min, before loading aliquots (5 μl) of desalted MUPs from the same *M. macedonicus* and *M. m. domesticus* samples as described previously. After loading, each sample was washed for 5 min, after which, the sample was eluted with a linear NaCl gradient of 0–500 mM in 30 min. Proteins eluting from the column were detected by monitoring the absorbance at 214 nm of the column eluate, in a flow cell of 1 cm path length

three *M. macedonicus* samples were characterized by a dominant mass peak at 18,742 Da (Fig. 3a). The close agreement between these three masses (0.4 Da) is within the expected instrument error (approximately ± 2 Da) and is consistent with all three samples containing the same, single protein species. The spectra all contained a second smaller peak at 18,723,

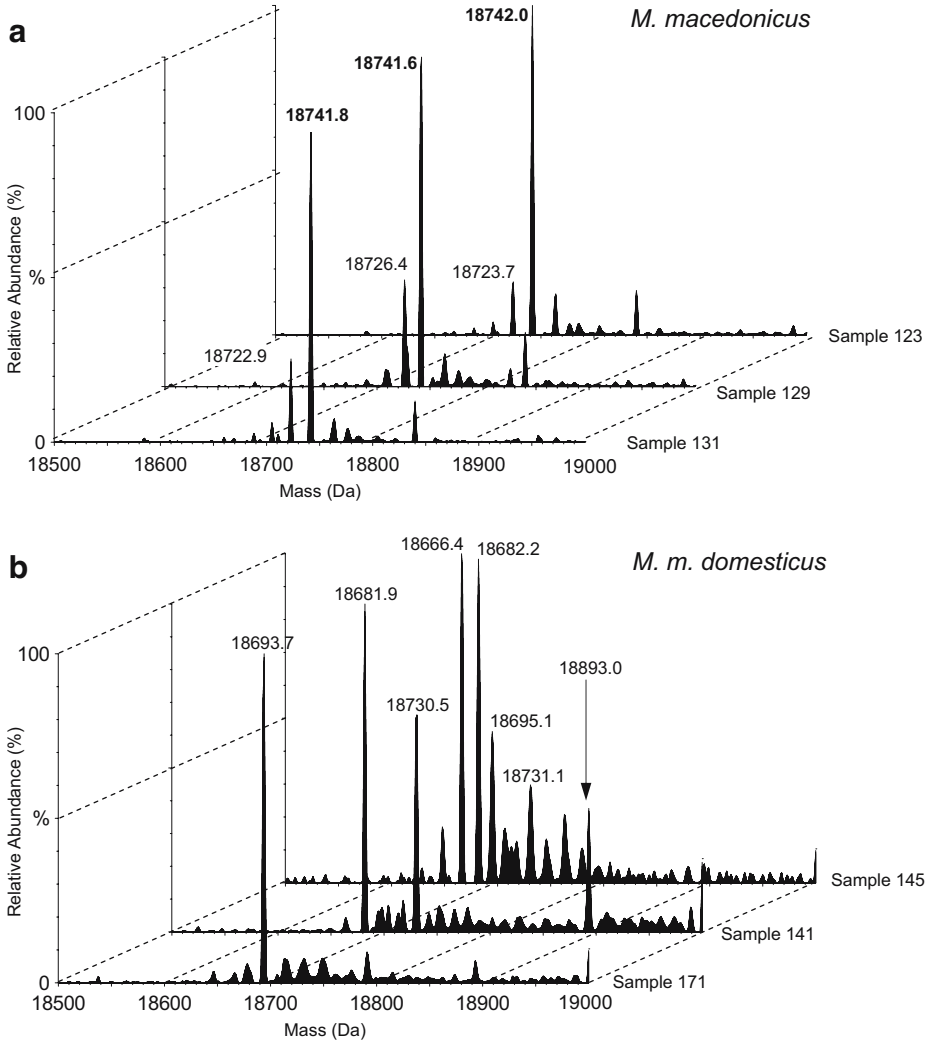


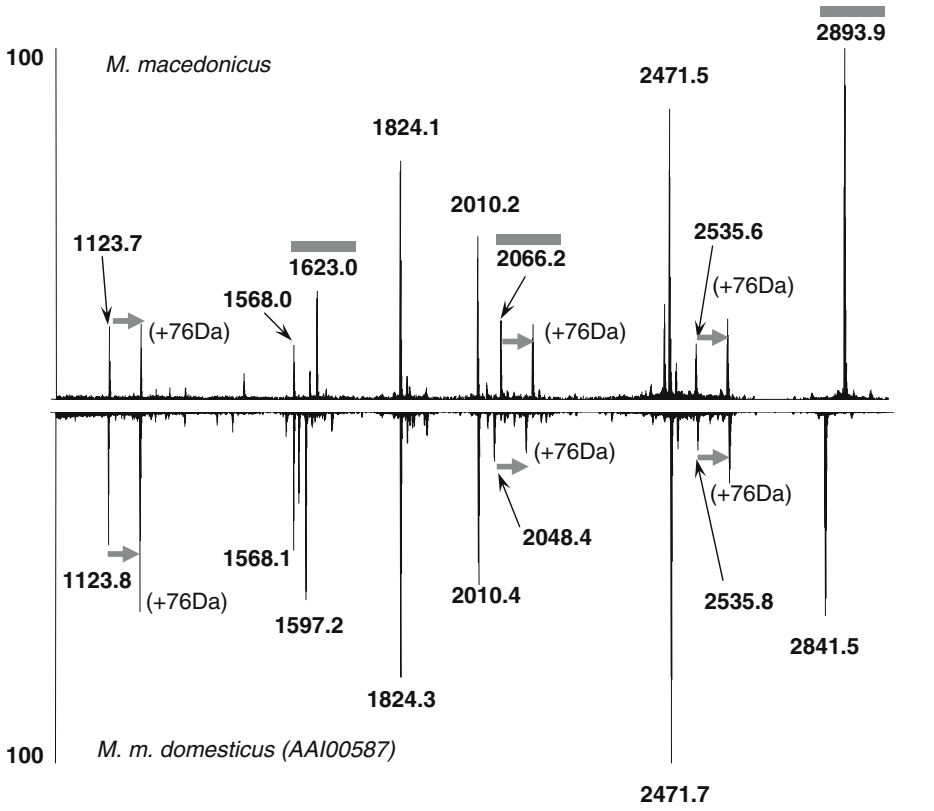
Fig. 3 ESI Mass Spectra of Unfractionated MUPs from three urine samples of (a) *Mus macedonicus* (123, 129, and 131) and (b) *M. m.domesticus* (141, 145, and 171). MUPs were separated (Fig. 2) and diluted 1:50 with a solution of 50% (v/v) acetonitrile/0.2% formic acid. This was introduced into a Q-tof Micro mass spectrometer (Micromass plc, Manchester UK) at 0.5 μl/min from a syringe infuser (Harvard Instruments, Holliston, MA, USA). The mass spectrometer was operated in TOF only mode, and raw data were gathered between m/z 700 and 1,400 with a scan/interscan time of 2.4/0.1 sec, respectively. Finally, raw data were deconvoluted and transformed to a true mass scale using MaxEnt 1 software

18,726, and 18,724 Da that probably also corresponds to a single protein. Two other features were also common to these mass spectra; a small peak at 18,763 Da and a slightly more abundant peak at 18,840 Da. The former, 21 Da higher than the base peak, is consistent with formation of a sodium adduct, whereas the latter is 98 Da higher than the base peak, consistent with the formation of a phosphate or sulfate adduct. We have commonly observed these artifacts in previous analyses, and it is unlikely that these reflect additional proteins.

The mass spectra obtained from urine samples of *M. m. domesticus* were more complex and heterogenous among individuals (Fig. 3b) than found for *M. macedonicus*, even though the two species were both collected from the same geographical region (Fig. 1). The number of major peaks (>20% base peak intensity) differed greatly. Sample 171 was the simplest, and unusually, expressed a single major peak at 18,694 Da, whereas sample 141 contained three peaks at 18,682, 18,730, and 18,893 Da. Sample 145 was most complex with peaks at 18,666, 18,682, 18,695, and 18,731 Da. Some of these masses have previously been observed in other wild mice or in inbred mouse strains, notably the protein at 18,694 Da, in C57BL/6, BALB/c, and wild mice (Robertson et al., 1996, 1997); 18,682 Da in wild mouse populations (Robertson et al., 1997), and 18,893 Da in inbred C57BL/6 mice (Armstrong et al., 2005).

Urine samples from *M. macedonicus* exhibited a single peak on anion exchange chromatography comprising a protein of mass 18,742 Da. To confirm that this protein was a MUP, and to investigate its relationship to known *M. m. domesticus* MUPs, the primary structure of the protein was analyzed by mass spectrometry. The protein was digested by endopeptidase Lys-C (Lys-C) in parallel with a MUP from urine of the inbred strain C57BL/6. Lys-C was selected over other proteases for this analysis as it was known from previous work to produce a small number of large peptides that were readily analyzed by MALDI-TOF mass spectrometry. This has previously allowed confirmation of >95% of the amino acid sequence from *M. m. domesticus* MUPs (Robertson et al., 1996). The amino acid sequence of the C57BL/6 reference MUP (GenBank accession number AAI00587, molecular mass 18,644 Da) had previously been inferred by comparison of intact protein molecular mass to the theoretical mass predicted by the cDNA sequence (Robertson et al., 1996) and comparison of Lys-C peptide masses to those predicted by the cDNA sequence (unpublished observation). This particular MUP was chosen on experimental grounds, as it was readily purified from C57BL/6 urine. The resultant peptides were surveyed by MALDI-TOF mass spectrometry. The mass spectrum of C57BL/6 MUP Lys-C peptides (Fig. 4), displayed nine masses corresponding to those predicted by the cDNA sequence (AAI00587). This analysis confirmed the identity of 156 of the 162 amino acids in the sequence (>96% coverage). The remaining unassigned amino acids were two tripeptides, the masses of which were too low for MALDI-TOF analysis. Three peptides (1123.8, 2048.4, and 2535.8 Th) were associated with additional "shadow" peaks at +76 Da. The predicted sequence for each of these peptides contains a cysteine residue that was maintained in the reduced state during proteolytic digestion by the addition of 2-mercaptoethanol. The 76-Da mass increase is consistent in mass with the formation of a β-mercaptoethanol adduct with those cysteine residues, providing good confirmation of the presence of cysteine residues in these peptides.

The corresponding mass spectrum of *M. macedonicus* Lys-C peptides revealed six peaks which shared masses with peptides in the C57BL/6 reference spectrum (Fig. 4). This was presumptive evidence for the identity of 100 amino acid residues and that the 18,742-Da *M. macedonicus* protein was indeed a MUP. The amino acid coverage in this instance was 61% (assuming a total of 162 residues). Three of the Lys-C peptides predicted from the cDNA sequence and present in the *M. m. domesticus* sample were absent from the *M. macedonicus* MUP (LysC peptides L2, L4, and L5), whereas the *M. macedonicus* sample contained three different peptides (m/z 1,623.0, 2,066.2, and 2,893.9). These peptides, likely to be the equivalent peptides to L2, L4, and L5, were selected for further MS analysis. The presence of a +76-Da adjacent peak (putatively a 2-mercaptoethanol adduct) to the peptide at m/z 2,066.2, suggested that in common with the equivalent *M. m. domesticus* peptide L5, it contained a cysteine residue.



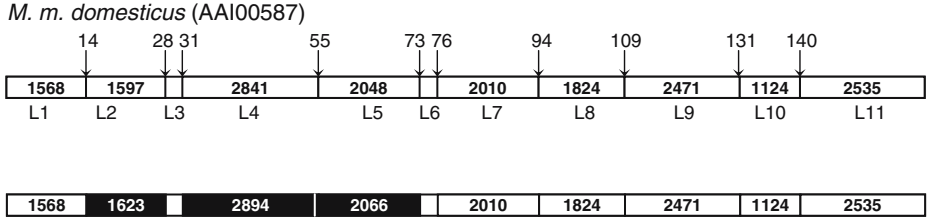


Fig. 4 MALDI-TOF peptide mapping of endoproteinase Lys-C peptides from a *Mus macedonicus* and *M. musculus domesticus* (C57BL/6) MUP. Anion exchange purified MUPs were digested with endoproteinase Lys-C (see Methods and Materials section for details). The mass of the resulting peptides was determined using MALDI-TOF mass spectrometry. Peptides were identified in the *M. m. domesticus* sample by comparison of the MALDI masses to a set of predicted masses (*L1–L11*) generated from a theoretical digest of a translated cDNA sequence (accession number AAI00587). Comparison of *M. macedonicus* peptide masses to those of *M. m. domesticus* allowed identification of both identical peptides and those where amino acid substitutions had occurred. Peptides thought to contain amino acid substitutions in the *M. macedonicus* sample are *labeled with a horizontal bar*. Additional ions, attributed to reaction of cysteine residues with β-mercaptoethanol, were observed 76 Da higher than certain peptides. Such peaks are labeled (+76 Da)

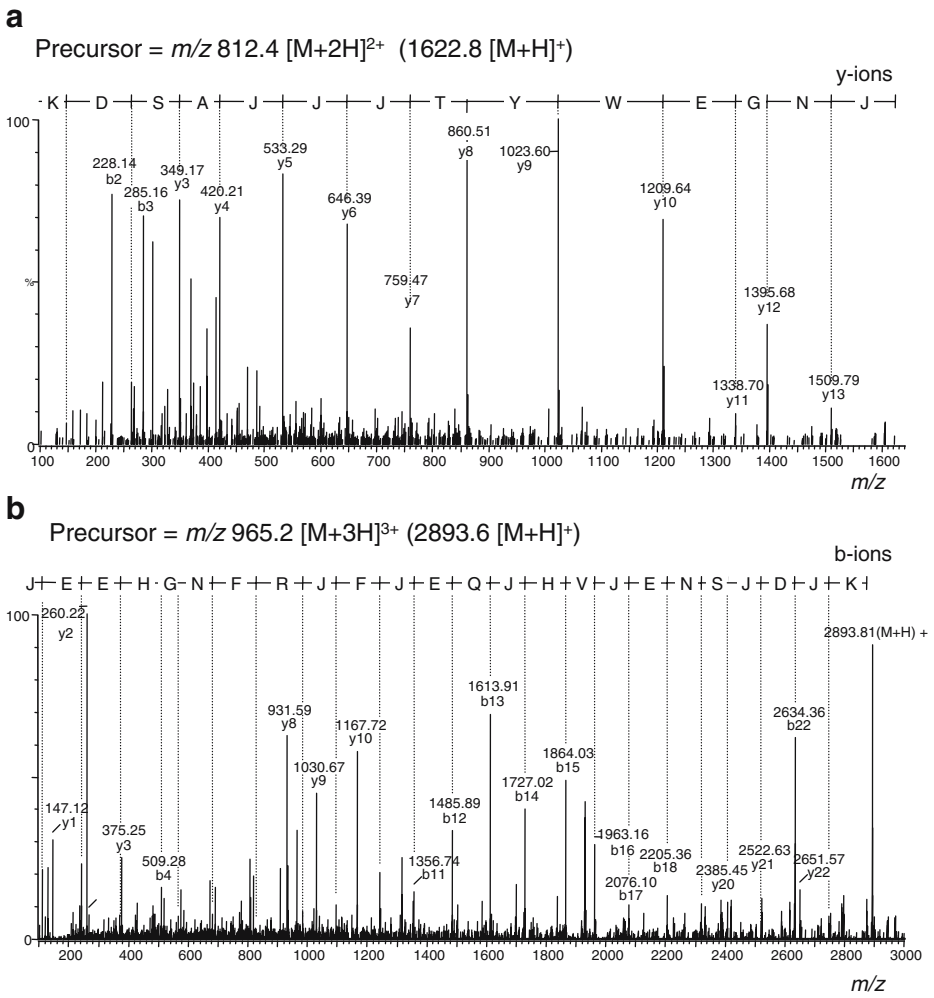


Fig. 5 Product ion mass spectra of (a) m/z 812.4 ($[M+2H]^{2+}$, 1,623.0 Da) and (b) m/z 965.2 ($[M+3H]^{3+}$, 2,893.9 Da) *Mus macedonicus* peptides. Tandem LC-MSMS experiments were performed on a Micromass Q-tof Micro mass spectrometer and a Dionex Ultimate RP-HPLC system (see Methods and Materials section for details). A Lys-C digestion of an anion exchange purified *M. macedonicus* MUP (18,742 Da) was introduced into the system, and ions of interest (m/z 812.4, 2+ and 965.2, 3+) were selected for tandem mass spectrometry using the data dependent switching feature within the MassLynx software. Fragmentation energy was similarly automatically selected using this feature. Product ion spectra were combined and de-convoluted using MaxEnt 3 software, with the charge state parameter determined from the isotope envelope of the precursor ion scan. De-convoluted spectra were interpreted with the MassSeq software included in the MassLynx package. Sequence information for the m/z 812.4 peptide was gained from a y-ion series, whereas that of the m/z 965.2 peptide was determined from a b-ion series. In this latter case, the identity of the 8 C-terminal residues was confirmed by a similar experiment on a trypsin digest (data not shown)

The three novel *M. macedonicus* peptides were sequenced by electrospray ionization tandem mass spectrometry. The first of the three unique *M. macedonicus* peptides (1,623.0 Th, $[M+H]^+$) was observed as a doubly charged ion at m/z 812.4 under electrospray ionization conditions. This fragmented readily to yield an abundant series of y ions (Fig. 5a). From these data, the amino acid sequence of the peptide was determined to be

*J*NGEWYT*JJJ*ASDK, where *J* is used to represent either leucine or isoleucine, which are isobaric and unable to be differentiated. This sequence is virtually identical to peptide L2 from *M. m. domesticus* AAI00587, ($_{15}$INGEWHTIILASDK$_{28}$), assuming correspondence of the leucine and isoleucine residues. The only difference between these peptides was the substitution of a tyrosine for a histidine residue in the *M. macedonicus* peptide. The mass difference of a His/Tyr substitution is +26 Da. The difference between the measured m/z values of the AAI00587 L2 peptide and the 1,623.0 *M. macedonicus* peptide is +25.8 Da, which confirms this as the sole change. In addition to characterizing this substitution, this experiment confirmed the identities of 14 additional amino acids in the *M. macedonicus* MUP, with the caveat of the residual ambiguity due to the isobaric pair of leucine and isoleucine.

The second unique *M. macedonicus* peptide (m/z 2,893.9 Th, $[M+H]^+$) was observed as a triply charged ion of m/z 965.2 in the precursor spectrum, implying an internal basic residue. During tandem mass spectrometry, this peptide did not generate a full series of y ions but fragmented to produce a complete set of b ions (Fig. 5b), from which the sequence of the peptide was determined as JEEHGNFRJFJEQJHVJENSJDJK. The sequence of the N-terminal eight residues of this peptide (JEEHGNFR) was confirmed by tandem mass spectrometry of a tryptic digest of the same preparation (data not shown). Although this sequence shared significant homology with peptide L4 from AAI00587, ($_{31}$IEDNGNFRLFLEQIHVLENSLVLK$_{55}$), it differed at three positions. The observed differences (from *M. m. domesticus* to *M. macedonicus*) were $D_{33}E$, $N_{34}H$, and $V_{53}D$. The arginine residue at position 40 was consistent with the triply charged precursor ion. Thus, an additional 24 residues of the *M. macedonicus* MUP were identified, and again, the interpretation was confirmed from the mass difference between the *M. m. domesticus* L4 peptide (52.4 Da) and the proposed *M. macedonicus* sequence (53 Da).

The final *M. macedonicus* peptide identified as a target for tandem mass spectrometry sequencing (2,066.2 Th, $[M+H]^+$) was also observed as a triply charged ion of m/z 689.4 under electrospray ionization conditions, consistent with an internal basic residue. Although this peptide did not generate a complete set of y or b ions (Fig. 6), the analysis yielded partial sequences from both y and b ion series that overlapped. The y ion series yielded the partial sequence of FHJ.....JSMVADK. The b ions gave a sequence of ...ECSEJSMV. Amalgamating these two sequences gave an overall amino acid sequence of FHJ...ECSEJSMVADK. When aligned to peptide L5 from AAI00587 ($_{56}$VHTVRDEECSELSMVADK$_{73}$), a high level of similarity was apparent. Two amino acid substitutions were observed in the *M. macedonicus* peptide; these were $V_{56}F$ and $T_{58}L$. The substitution of F/V at position 56 has been previously observed in other MUPs, and the V_{56} residue in the C57BL/6 sequence used in this study (AAI00587) is only observed in a minority of cases. This residue is located within the central, ligand-binding calyx and modulates the ability to bind specific ligands (Darwish Marie et al., 2001).

Four residues in the middle of the peptide remained unassigned. To establish the identity of these residues in L5, a Lys-C digest of the *M. macedonicus* protein was treated with an ethanolic solution of acetyl chloride to convert free carboxyl groups within the peptides to the corresponding methyl esters. Peptides, thus modified, increase in mass by 14 Da for each methyl group added, quantifying the number of acidic residues within the peptide. MALDI-TOF mass spectrometry of a reduced, carbamidomethylated and esterified Lys-C digestion of the *M. macedonicus* MUP (Fig. 7) yielded ions of masses consistent with the predicted number of carboxyl groups from previous assignments. For peptide L5, the mass difference between the carbamido peptide and the methylated carbamido peptide was 84 Da (6×14), which identified five acidic residues plus the C terminus. Previous tandem mass

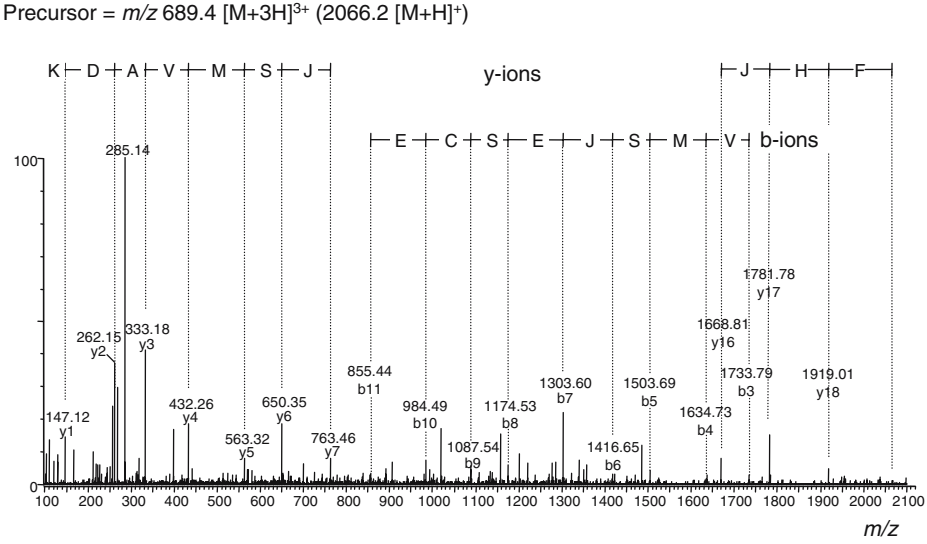


Fig. 6 Product ion mass spectrum of m/z 689.4 ($M+H^+$ 2,066.2 Da) *Mus macedonicus* peptide. A tandem LC-MSMS experiment was performed on a *M. macedonicus* Lys-C peptide of m/z 689.4, under identical conditions to those described in the Fig. 5 legend. Once again, this peptide was isolated from a *M. macedonicus* MUP Lys-C digest, using data dependent switching. The MaxEnt 3 de-convoluted product ion spectrum was interpreted using PepSeq software. In this instance, a composite partial sequence was inferred from both b and y ions

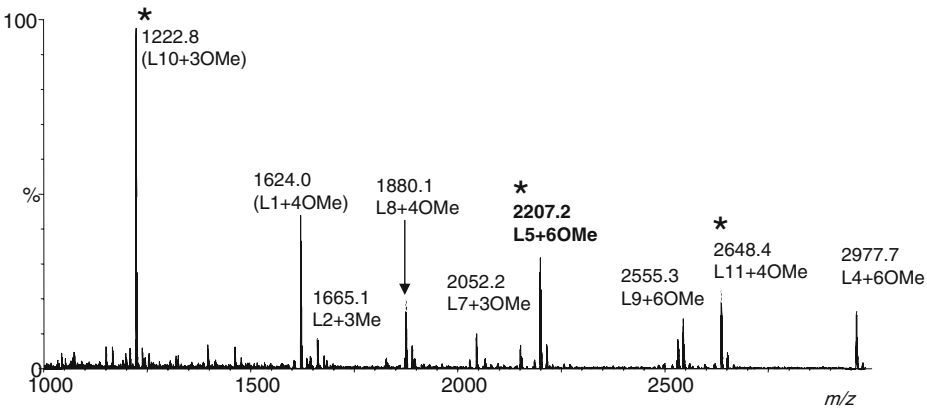


Fig. 7 MALDI-TOF mass spectrum of a methyl esterified Lys-C digest of a carbamidomethylated *M. macedonicus* MUP. Quantification of acidic residues within Lys-C peptides from a *M. macedonicus* MUP was achieved by methyl esterification. Before digestion, an anion exchange purified *M. macedonicus* MUP preparation was reduced, carbamidomethylated, and digested with Lys-C (see Methods and Materials). Peptides were then extracted from the solution using reverse phase chromatography media (Zip Tips), washed with 0.1% (v/v) trifluoroacetic acid (TFA), and finally desorbed and collected in a solution of 50% (v/v) acetonitrile/0.1% (v/v) TFA. Solvents and TFA were then removed from this preparation using a vacuum centrifuge. A 1-ml aliquot of methanol was incubated at −20°C for 15 min before addition of acetyl chloride (150 µl). A 20-µl aliquot of this solution was then added to the dried Lys-C peptides and incubated at RT for 45 min. Esterified Lys-C peptides unique to *M. macedonicus* are marked with an asterisk (*)

spectrometric analysis of this peptide identified the positions of three of these residues, leading to the conclusion that two of the four unidentified residues were also acidic, namely, aspartate (D) or glutamate (E).

The identification of two acidic residues in the unassigned portion of L5 allows inferences to be drawn as to the identities of the remaining two unassigned residues. On ESI-MS, peptide L5 is triply charged, implying that it contains an internal basic residue. Although the histidine residue at position 57 is weakly basic, a more likely explanation of this charge state is that the arginine residue at position 60 in the *M. m. domesticus* sequence is conserved, as observed in all the urinary MUPs previously characterized in our laboratories (Robertson et al., 1996). The L5 peptide from *M. m. domesticus* is 42 Da heavier than the equivalent peptide from *M. macedonicus* (allowing for residues assigned by tandem mass spectrometry). The assumption that R_{60} is conserved and the knowledge that two of the residues are acidic allows for only two possibilities for the final assignment of the sequence. In the first scenario, the two acidic residues at positions 61 and 62 are identical to the *M. m. domesticus* sequence, in which case a V/G substitution at position 59 in the *M. macedonicus* sequence would account for the mass difference. The second scenario allows for the possibility of a D/E substitution at position 61 and a V/A substitution at position 59 to give a net loss of 42 Da in the *M. macedonicus* peptide. On the basis of this analysis, the remaining four amino acid residues in L5 are assigned either as [GR(DE)] or [AR(DD)]. The order of these amino acids is not apparent from the above analysis and has been assigned on the basis of the overall level of conservation observed between the two MUPs (Fig. 8).

Endopeptidase Lys-C digestion of AAI00587 creates two tripeptides (L3 and L6), each of which has a predicted mass of less than 400 Da. Such peptides are difficult to analyze with MALDI-TOF mass spectrometry because of background signal from ions in the chemical matrix. We have, however, observed in previous experiments that Lys-C has a

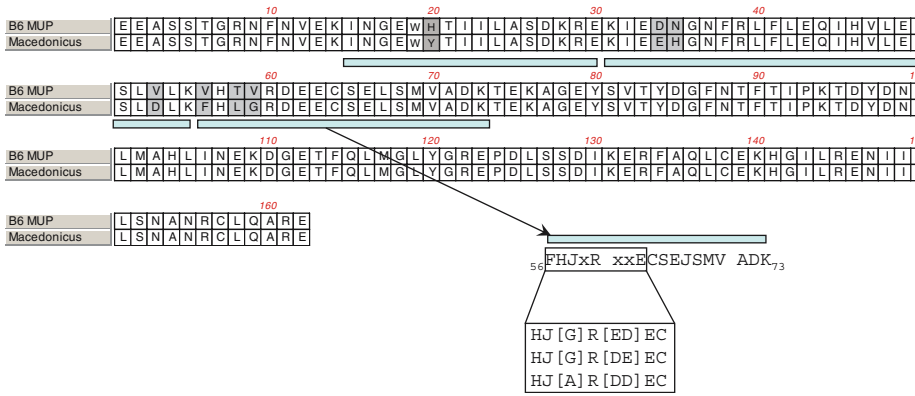


Fig. 8 Comparison of *M. macedonicus* and *M. m. domesticus* MUP sequences. Inferred data about the sequence of the *M. macedonicus* MUP from MALDI-TOF, LC-MS, and LC-MSMS analysis were amalgamated into a single composite sequence and compared to the known sequence of a *M. m. domesticus* MUP (accession number AAI00587, labeled "B6 MUP"). Positions of amino acid substitutions in the two sequences are shaded. Full sequence identification was not achieved between residues 59 and 62, but was narrowed down to three permutations following methyl esterification (see Fig. 7). An expanded view of this area is inset and the three possible permutations included. Amino acid identities within the three permutation sequences inferred from methyl esterification are shown in brackets. The arginine residue at position 60 is assigned provisionally, pending further proof, on the basis of overall peptide charge state (3+) and conservation between known MUP sequences

tendency to omit cleavages in the peptide chain in areas where lysine residues are in close proximity. To assess whether the two tripeptides in *M. macedonicus* possessed the same sequence as those in AAI00587, an additional LC-MS experiment was run on the Lys-C peptides. In this instance, the m/z values for L2+L3 (1,018.54, $[M+2H]^{2+}$) and L5+L6 (820.70, $[M+3H]^{3+}$ with an oxidized methionine residue) were readily observed in extracted ion chromatograms (data not shown). The two tripeptides were therefore assigned to the *M. macedonicus* sequence as identical to the *M. m. domesticus* sequence.

The assembled *M. macedonicus* sequence was aligned to that of AAI00587 (Fig. 8). Of the 162 amino acids in the *M. macedonicus* sequence, 152 are identical to AAI00587, seven residues are known to differ, and the identity of a further three is unclear, although their chemical class has been determined. This has allowed the modeling of the variant residues into the three dimensional structure using the Modeler facility within the Discovery software package and crystallographic coordinates (Kuser et al., 2001) (Fig. 9), lodged in the National Centre for Biotechnology Information (NCBI) structural database (http://www.ncbi.nlm.nih.gov).

In constructing the model *M. macedonicus* MUP structure, we had to consider two types of uncertainty in the sequences derived from proteomics experiments. First, the amino acids between residues 58 and 61 could not be unambiguously determined by tandem mass spectrometry, and the three possibilities that existed (GRED, GRDE, ARDD) were modeled. Secondly, it is not possible to discriminate between the isobaric residues leucine and isoleucine. To assess the importance of these residues, we constructed two artificial sequences based on AAI00587, in which all Ile/Leu residues were converted to either Leu or Ile. Models built on the parent 1I04 structure were compared, and the RMSD of the alpha carbon atoms of the two structures was 0.14 Å. The overall main chain trajectories were virtually identical. We conclude that Leu/Ile substitution would not have a major effect on the model structures. Accordingly, we built *M. macedonicus* models assuming that the identity of Leu/Ile residues were the same as for AAI00587. For the six variant structures based on the unresolved pentapeptide (see above), all yielded high quality models with alpha carbon RMSD of less than 0.25 Å and similar main chain trajectories (Fig. 9). All proteins passed the Protein Health checks built into the Modeler package. Other than

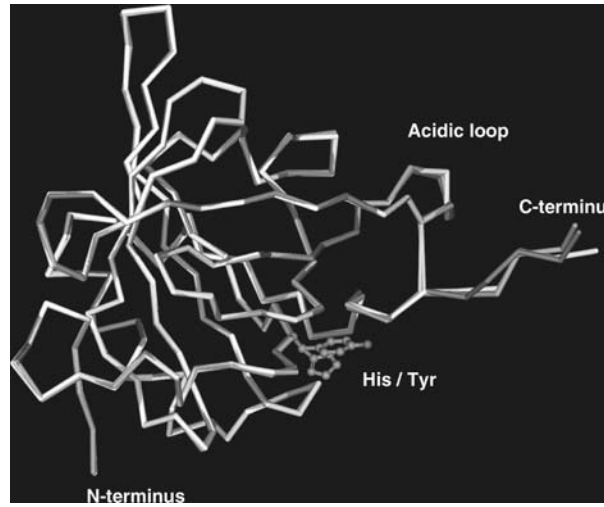


Fig. 9 Homology model building of *M. macedonicus* sequences. The three variants of the possible *M. macedonicus* sequence, varying at the acidic loop (GRED, GRDE, and ARDD) were used to construct homology models based on the MUP structure 1I04.PDB. All three models were aligned structurally to the 1I04 structure, with an RMSD in each instance of less than 0.16 Å. The uncertain acidic loop is highlighted, as is the His/Tyr substitution that is in close proximity to the GxW lipocalin motif. Because the structures are so similar, no labels have been included to identify them individually

the ambiguous acidic loop region of the *M. macedonicus* protein sequences, there are a number of amino acid substitutions relative to MUPs from *M. m. domesticus*. These changes ($H_{20}Y$, $D_{34}E$, $N_{35}H$, $V_{53}D$, $T_{58}L$, $V_{59}G$) do not influence the ligand binding cavity and are solvent exposed.

To assess the level of similarity between the *M. macedonicus* sequence and those from *M. m. domesticus*, we compared it to all the known *M. m domesticus* sequences in the NCBI database. The amino acid sequence of the *M. macedonicus* MUP shares most similarity with a group of MUPs for which only incomplete sequences are available. These, in turn, are most similar to the male-specific 18,893 Da MUP discovered in C57BL/6 mouse, but also widely present in wild caught *M. m. domesticus*. This protein has a high capacity for binding of the male specific ligand 2-*sec*-butyl-4,5-dihydrothiazole (thiazole) that accords with a function in male-specific signaling (Armstrong et al., 2005).

The simple MUP pattern in *M. macedonicus*, together with the lack of individual variability, suggests that MUPs do not have sufficient polymorphism to provide an individual ownership signal in scent marks in this species, in contrast to *M. m. domesticus*. This may reflect the difference in the population ecology of this aboriginal grassland species compared to the commensal house mice and possibly reflects the more ancestral form. While the field ecology of *M. macedonicus* is not well known, in line with the other aboriginal species of *Mus* in Europe and the Middle East, *M. macedonicus* in Turkey can be presumed to have large territories, and individuals are highly agonistic to each other (reviewed by Frynta et al., 2005). Both male and female *M. macedonicus* are much more aggressive than *M. m. domesticus* (Frynta and Čiháková, 1996), and individuals may be largely nonoverlapping. By contrast, in commensal *M. m. domesticus* populations, multiple males and females live within territorial social groups, and there may be extensive spatial overlap between neighbors when borders are not easily defended (Hurst, 1987; Barnard et al., 1991). This results in an unusually high level of aggregation and contact between individuals in this species. As male *M. m. domesticus* advertise their territorial dominance through scent marks (Hurst and Beynon, 2004), it is possible that this may have been a strong driver for the evolution of individual-specific MUP patterns in *M. m. domesticus* but not in the aboriginal species. Given their dispersed distribution, there is likely to be much less requirement for advertising individual scent ownership in *M. macedonicus* and other widely dispersed rodent species. This suggests the intriguing hypothesis that the extreme polymorphism and individual variability in MUP patterns seen in *M. m. domesticus* is a species-specific adaptation for signaling individuality in a complex social system where individuals vary in social status. *M. macedonicus* is one of a number of aboriginal mouse species that have evolved independently of *M. m. domesticus*, inhabit different environments, and display different forms of behavior. Information about the MUPs and MUP genes in these animals remains scarce (Sampsell and Held, 1985), yet they provide a valuable opportunity to investigate the function of both MUPs and their ligands and the evolution of a family of proteins used for scent signaling. To this end, a wider survey of MUPs from aboriginal mice of both sexes, which includes both primary structure and ligand status, has the possibility to produce greater insights into the wider context of MUP polymorphism and function.

Acknowledgments We are grateful for the assistance of Coskun Tez and support from the Genetics Society toward the fieldwork. The work was carried out under research grants from the Biotechnology and Biological Sciences Research Council, UK to J.L.H. and R.J.B.
In memory of J.E. Robertson, 1928–2003.

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J Chem Ecol (2007) 33:631–642
DOI 10.1007/s10886-006-9249-y

Qualitative Analysis of Red Imported Fire Ant Nests Constructed in Silica Gel

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Received: 22 July 2006 / Revised: 15 November 2006 / Accepted: 16 December 2006 /
Published online: 25 January 2007
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Abstract Red imported fire ants, *Solenopsis invicta* Buren, build nests by excavating soil. Incorporation of ant-derived chemicals in nesting material has long been known; however, only a few chemicals have been identified. This paucity of identified ant-derived chemicals may be due to the interference from soil-borne compounds in chemical analysis. In the laboratory, red imported fire ants were able to build their nest using moistened silica gel as the only building material. This provided an opportunity to establish a profile of ant-derived chemicals in nest material without the presence of any soil-borne artifacts. A new method for profiling ant-derived chemicals in nest material by using GC-MS was developed. All nests contained cuticular hydrocarbons and venom alkaloids. Phosphoric acid, glycerol, lactic acid, and malonic acid also were identified from samples collected from the silica gel nest.

Keywords *Solenopsis invicta* Buren · Nest · Cuticular hydrocarbons · Venom alkaloids · Organic acids · Amino acids · Phosphoric acid · Glycerol

Introduction

The red imported fire ant, *Solenopsis invicta* Buren, is a nest-building ant species that builds its nests by excavating soil. An ant nest not only protects the colony from intruders, but also provides a system for microclimate regulation, which is essential for ant colony survival (Hölldobler and Wilson, 1990). Nest construction by *S. invicta* not only alters the physical properties of the soil, such as infiltration and leaching (Green et al., 1998, 1999), but also the chemical properties, such as element enrichment (Herzog et al., 1976; Lockaby and Adams, 1985; Green et al., 1998, 1999). The ant nest is a central site for many ant activities, and chemicals in the nest may influence colony activities. There is evidence

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that red imported fire ants incorporate ant-derived organic compounds into the nest soil. For example, cuticular hydrocarbons were found in the nest soil (Vander Meer, unpublished data, cited in Vander Meer and Lofgren, 1988). Red imported fire ants also have been reported to disperse venom alkaloids on the brood surface and mound soil as disinfectants (Obin and Vander Meer, 1985; Storey et al., 1991).

Ants have been reported to be able to differentiate their own nests from others. For example, Florida harvest ants, *Pogonomyrmex badius* (Latreille), showed a strong preference towards their own nest material when presented a choice between their own nest material and that of another colony (Hangartner et al., 1970). Hubbard (1974) found fire ants preferentially dug in nest materials from their own colony when offered a choice of soil from their own mound and fresh soil or nest material from another colony. Singer and Espelie (1998) found that workers of fungus-growing ants, *Apterostigma collare* Emery, differentiated their own nest from other nests, and suggested that chemicals incorporated into the nest soil might be the basis for nest recognition.

An ant nest should be an ideal place for the growth of microorganisms, considering the regulated moisture and temperature conditions, and a mechanism to suppress the growth of pathogenic microorganisms inside the nest may be critical to colony fitness. Incorporating antimicrobial agents into nesting material may serve this purpose. Indeed, venom alkaloids of fire ants are known antimicrobial agents (Blum, 1988; Storey et al., 1991).

Soil is the most complex biomaterial on earth (Young and Crawford, 2004), and chemical analysis of organic components in soil is often problematic. In a preliminary study, I found that red imported fire ants would build their nests using moistened silica gel as the only building material under laboratory conditions (Chen, unpublished data). This provided an opportunity to analyze the chemicals incorporated in the nest material by red imported fire ants without interference from any soil-borne chemicals. The objective of this study was to establish a detailed profile of ant-derived chemicals in the nesting material by extraction and analysis of nests constructed from silica gel.

Methods and Materials

Collection and Maintenance of Colonies Ant colonies were collected from Washington County, Mississippi, USA, and the water drip method was used to separate ants from mound soil (Banks et al., 1981). Ants were maintained in a 44.5×60.0×13.0-cm plastic tray. Fluon® (Ag Fluoropolymers, Chadds Ford, PA, USA) was coated on the inside wall of the tray to prevent ants from escaping. Distilled water, 10% sugar solution, and adult crickets were provided to colonies *ad libitum*. A 14×2.0-cm Petri dish with 1.0 cm of hardened dental plaster (Castone®, Dentsply International Inc. York, PA, USA) on the bottom was used as an artificial nest, placed in the center of each tray. There was a 5-cm diam brood chamber in the bottom of the Petri dish. Above the dental plaster were two 8-mm access holes. The Petri dish lid was painted black (1302 Gloss Black Spray Enamel, Louisville, KY, USA) to block light. All colonies were maintained at 21–27°C and RH between 70 and 85%. The social form of *S. invicta* colonies was determined using PCR. Primers described in Valles and Porter (2003) were used to amplify *Gp-9* alleles indicating monogyne or polygyne colony status. Specimen collection, DNA extraction, and PCR methods were as described by Chen and Allen (2006).

Nesting Devices and Procedures Fire ant workers exhibited nest building behavior even when the queen was absent. Two nest-building scenarios, queenless subset colonies and queenright intact colonies, were established in the laboratory by using silica gel (35–60 mesh, Sigma-Aldrich, St Louis, MO, USA) as the only nest building substrate. The device for a queenright intact colony scenario consisted of one plastic tray (20×6 cm) with a 500-ml square bottle right underneath it. There were five 3-mm access holes between the tray and the bottle, and the inside wall of the tray was coated with Fluon®. The bottle was filled with moistened silica gel (50% water, *w*/*w*), which had been washed with acetone and distilled water and dried at 350°C for at least 12 hr. The nesting device for the queenless scenario was similar except smaller in size. A petri dish (8.6×2.2 cm) and a capped Wheaton liquid scintillation vial (20 ml) were used. The inside wall of the Petri dish was coated with Fluon®. There was a 3-mm access hole between the Petri dish and the vial.

Mature colonies were used in this experiment as demonstrated by the presence of brood and alates. Four colonies (three polygyne and one monogyne) were used for the queenright colony scenario and seven colonies (five polygyne and two monogyne) for the queenless scenario. The entire colony was used for each queenright scenario (21 to 45 g), whereas in the queenless scenario, 1.25–1.50 g ants [12.2–18.2% brood and 8.2–10.0% alates (male + female)] were used for each nesting device. Ants were released in the nesting device. All experiments were conducted at 21–27°C and RH between 70 and 85%. Controls were established in the same manner as described except no ants were released into the devices. No food was provided in nesting devices for either treatments or controls.

Collection of Silica Gel Samples Ants removed silica gel from the bottle and vials when they built their nests. While ants were making their nest, the silica gel removed was accumulated in the plastic tray of the queenright scenario or the Petri dish in the queenless scenario. After 2 wk, these silica gel samples were collected by directly transferring silica gel from the nest devices into glass beakers. No further separation of ants from silica gel was necessary because at that time only a few ants were usually present, which were removed with forceps.

Silica gel samples in the nest (inside the bottles or vials) were collected in a different way. Because most ants were inside the nest, they needed to be separated from the silica gel. The nest sand was collected by first disassembling the nest device and transferring the silica gel and ants into an aluminum pan (33.5×6.5 cm) with its inside wall coated with Fluon®. The silica gel was then spread on the bottom of the pan. A Petri dish (8.6×2.2 cm) with a 0.5-cm entrance hole on the side was placed upside down in the pan and served as an artificial nest. Ants moved into the Petri dish with all their brood avoiding the open space. The silica gel in the pan was collected after workers with all the brood had moved into the artificial nest. Samples were stored at −15°C in a glass beaker, which was sealed with Teflon tape. Only fresh samples, stored in the refrigerator for less than 2 d, were used for chemical analysis.

Extraction Some silica gel particles in the plastic tray and Petri dish were colored either brown or brownish–yellow, which was attributed to ant excretion. The colored silica gel samples (each consisted of 10–15 silica gel particles) were directly derivatized with 50 µl *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 150 µl pyridine at 60°C for 6 hr. Uncolored silica gel from outside the nest and silica gel inside the nesting device (each sample consisted of 2.5–3.0 g silica gel) were analyzed separately, and all these silica gel samples were subjected to three different solvent extractions: pentane, pyridine, and water

(6×3 ml). Pentane and pyridine extractions were concentrated to 200 μl and no derivatization was conducted. The water extract was freeze-dried and then derivatized by using 50 μl BSTFA in 150-μl pyridine at 60°C for 6 hr.

Chemical Identification The presence of cuticular hydrocarbons and venom alkaloids in nesting material was confirmed by comparing chromatograms and mass spectra of silica gel samples to those of worker pentane extractions. Both cuticular hydrocarbon and venom alkaloids have been extensively analyzed by using gas chromatography. Because the amounts of cuticular hydrocarbons and venom alkaloids were both in the microgram range, the profiles were well established (Nelson et al., 1980; Obin, 1986; Ross et al., 1987). Five signature cuticular hydrocarbon peaks and four of the most significant venom alkaloid peaks were used in this study because the patterns of these peaks had been previously identified (Vander Meer and Lofgren, 1988). Cuticular hydrocarbons included n-heptacosane, 13-methylheptacosane, 13, 15-dimethylheptacosane, 3-methylheptacosane, and 3,9-dimethylheptacosane (Obin, 1986), and four venom alkaloids included *trans*-2-methyl-6-(*cis*-4-tridecenyl)piperidine, and *trans*-2-methyl-6-n-tridecylpiperidine, *trans*-2-methyl-6-(*cis*-6-pentadecenyl)piperidine, and *trans*-2-methyl-6-n-pentadecylpiperidine (Vander Meer, 1986; Ross et al., 1987). Cuticular hydrocarbons and venom alkaloids in worker pentane extraction were separated with a silica gel column consisting of a 14.6× 0.6 cm disposable pasteur pipette filled with 5 cm 35–60 mesh silica gel with the tip blocked with a plug of glass wool. One gram of workers was extracted in 5-ml hexane for 5 hr and the extraction was concentrated to 200 μl under N_2 flow and then loaded onto the silica gel column. Cuticular hydrocarbons were eluted with the first 8-ml hexane, and venom alkaloids were then eluted with 4-ml pyridine. Identification of all other chemicals was achieved by comparing the GC retention time and mass spectrum of targeted compound to those of chemical standards.

Gas Chromatography–Mass Spectrometry A Varian GC-MS system was used consisting of a CP-3800 gas chromatograph with a DB-1 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) and a Saturn 2000 mass selective detector. The GC temperature was programmed as follows: initial temperature 50°C, held for 1 min, increased to 240°C at 20°C/min, and held for 29.5 min. The split ratio was 1:10, injection temperature was 250°C, and transfer line temperature was 270°C. The mass spectrometer was operated at 70 eV in the electron impact mode.

Results

Ants removed silica gel from the bottles or vials until the space in the bottle or vial could accommodate the whole colony. Workers moved all brood into the bottles or vials within 2 d. Workers preferred depositing their excreta along the edge of the nesting device. Some silica gel particles turned either brownish or yellow, most likely due to excretion. After ants built their nest in the nesting devices, only a few workers were seen outside the nest. Cuticular hydrocarbons were found in pentane and pyridine extracts of all samples, and the retention times and mass spectra of cuticular hydrocarbons matched those of worker body extractions (Fig. 1). Venom alkaloids were found in pyridine extracts of all samples collected inside the nest; however, in only one sample (queenless), four venom alkaloids were all detected. No venom alkaloids were

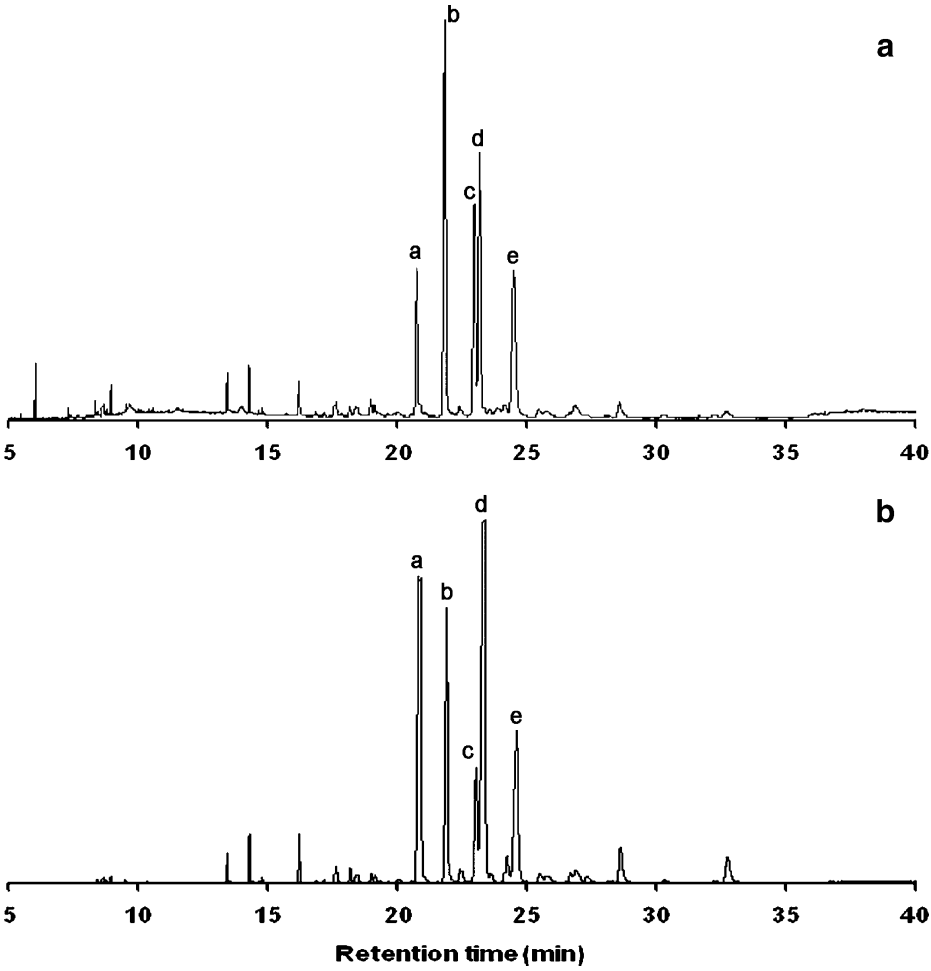
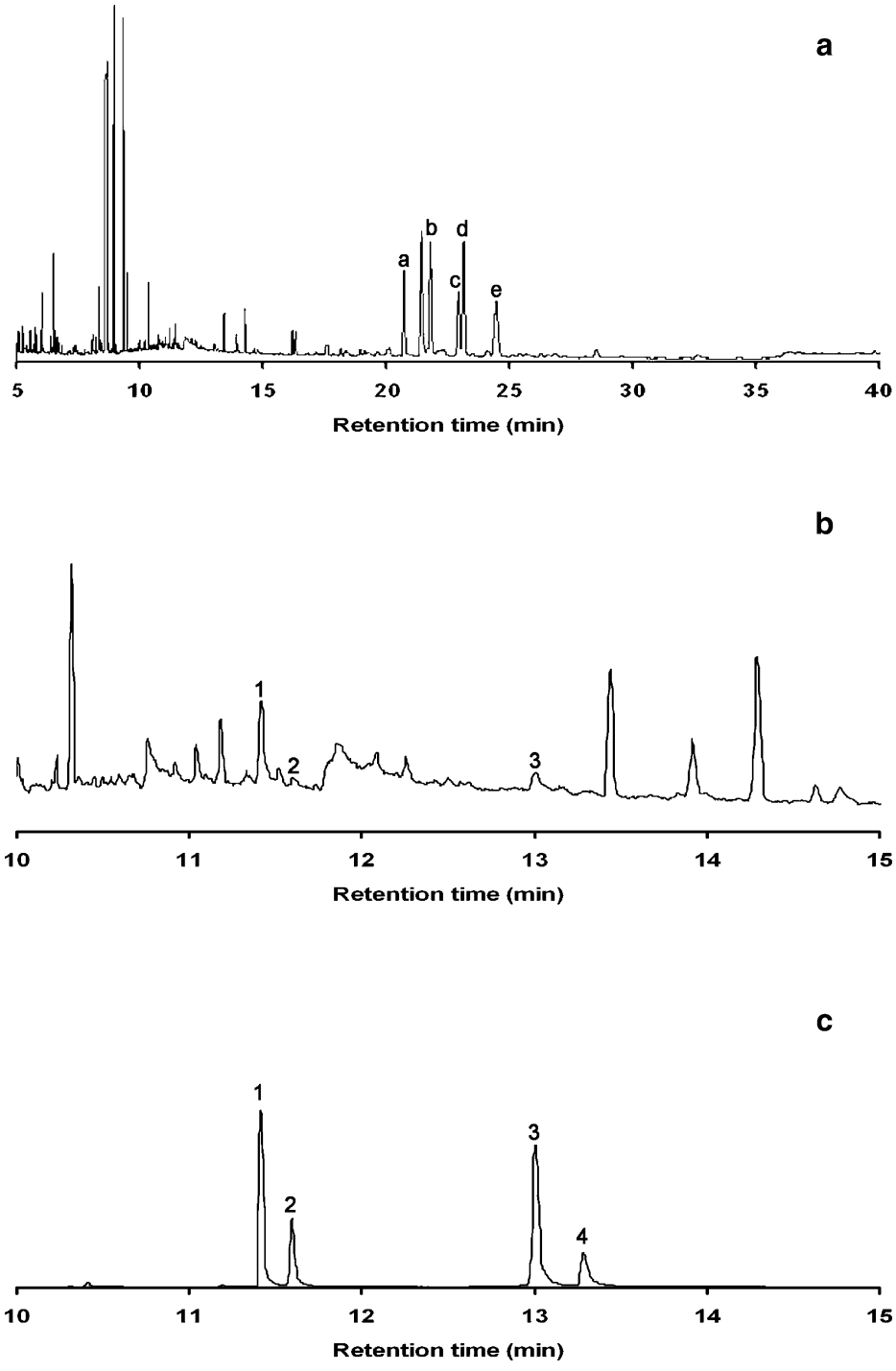


Fig. 1 Typical GC-MS total ion chromatograms of pentane extracts of silica gel samples (**a**) and cuticular hydrocarbons extracted from workers (**b**). Cuticular hydrocarbons were detected in all silica gel samples from nesting devices. Peak assignment: a: n-heptacosane; b: 13-methylheptacosane; c: 13, 15-dimethylheptacosane; d: 3-methylheptacosane; e: 3,9-dimethylheptacosane

found in silica gel, which was removed from the nest by ants while they were building their nest. The retention times and mass spectra of venom alkaloids matched those of worker body extractions (Fig. 2). The water extract of silica gel collected inside the nests contained lactic, malonic, and phosphoric acids as well as glycerol (Fig. 3).

Uric acid, urea, glycerol, phosphoric acid, amino acids, and organic acids were found in silica gel particles with ant excretion (Fig. 4). Thirteen organic acids were identified from all excreta samples, including lactic, malonic, benzoic, succinic, methylmaleic, 3-methylglutaric, malic, suberic, azelaic, hexadecanoic, 2-quinolinecarboxylic, gluconic, and stearic acids. Nine amino acids were identified in excreta samples, including alanine, glycine, *N*-acetylglycine, serine, threonine, aspartic acid, glutamine, phenylalanine, and tyrosine. The profile of amino acids in excreta was not consistent among


a
a
b
c
d
e
5
10
15
20
25
30
35
40
Retention time (min)
b
1
2
3
10
11
12
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14
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Retention time (min)
c
1
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Retention time (min)

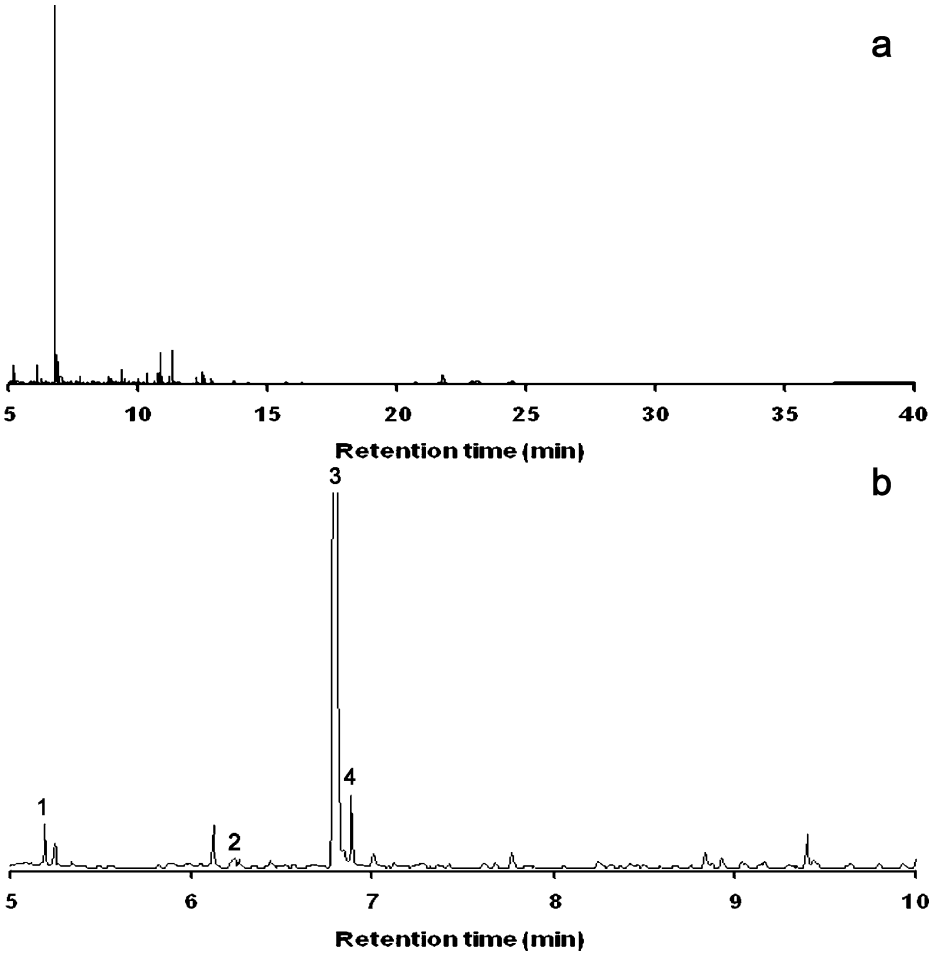


Fig. 3 Typical GC-MS total ion chromatograms of water extracts of silica gel samples collected inside the nest (**a**). Chromatogram between retention times 5–10 min was shown (**b**) to highlight identified chemicals. Peak assignment: 1: lactic acid-tms; 2: malonic acid-tms; 3: phosphoric acid-tms; and 4: glycerol-tms

colonies, except for *N*-acetylglycine, which showed up in every excreta sample. The chemical profile of the ant excretion was by no means complete because numerous peaks have not been identified (Fig. 4).

All identified compounds, along with their sample types, analysis methods, and the sources of authentic standards are summarized in Table 1.

◀**Fig. 2** Typical GC-MS total ion chromatograms of pyridine extracts of silica gel samples collected inside the nest (**a** and **b**, **b** was used to highlight the same chromatogram at retention times from 10–15 min) and venom alkaloids in worker extraction (**c**). Peak assignment: a: n-heptacosane; b: 13-methylheptacosane; c: 13, 15-dimethylheptacosane; d: 3-methylheptacosane; e: 3,9-dimethylheptacosane; 1: *trans*-2-methyl-6-(*cis*-4-tridecenyl)piperidine; 2: *trans*-2-methyl-6-n-tridecylpiperidine; 3: *trans*-2-methyl-6-(*cis*-6-pentadecenyl) piperidine; 4: *trans*-2-methyl-6-n-pentadecylpiperidine

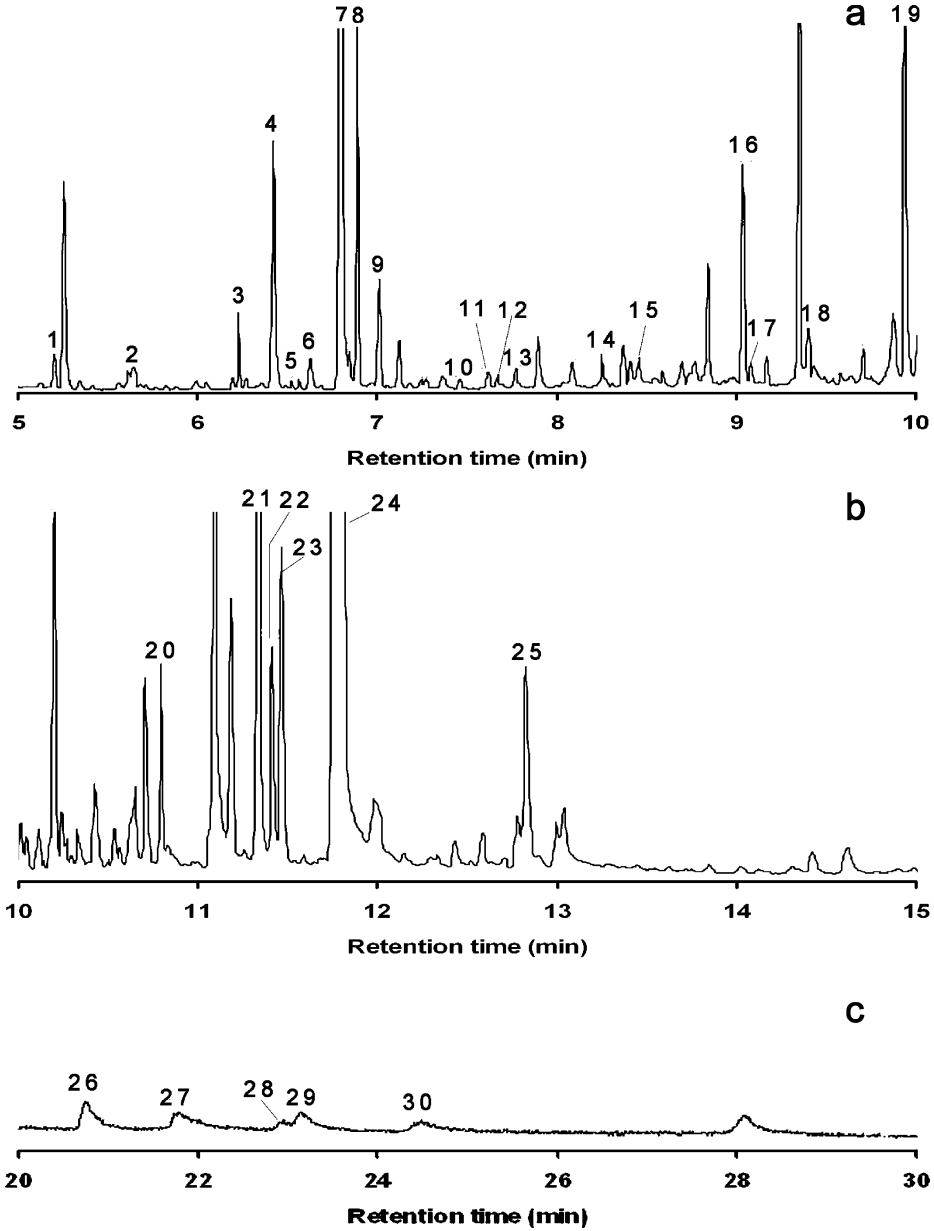


Fig. 4 Typical GC-MS total ion chromatograms of derivatized extracts of colored silica gel particles (excreta). Chromatogram between retention time 5–10 min was shown in **a**, 10–15 min in **b**, and 20–30 min in **c**. Chromatogram for retention 15–20 min was not shown because no peaks were identified. Peaks assignment: 1: lactic acid-tms; 2: glycine-tms; 3: malonic acid-tms; 4: urea-tms; 5: benzoic acid-tms; 6: *N*-acetylglycine-tms; 7: phosphoric acid-tms; 8: glycerol-tms; 9: succinic acid-tms; 10: serine-tms; 11: methylmaleic acid-tms; 12: threonine-tms; 13: 3-methylglutaric acid-tms; 14: malic acid-tms: 15: aspartic acid-tms; 16: glutamine-tms; 17: phenylalanine-tms; 18: suberic acid-tms; 19: azelaic acid-tms; 20: tyrosine-tms; 21: hexadecanoic acid-tms; 22: 2-quinolinecarboxylic acid-tms; 23: gluconic acid-tms; 24: uric acid-tms; 25: stearic acid-tms; 26: n-heptacosane; 27: 13-methylheptacosane; 28: 13, 15-dimethylheptacosane; 29: 3-methylheptacosane; 30: 3,9-dimethylheptacosane

Table 1 Profile of ant-derived compounds in silica gel nesting material

Sample Type	Analysis Method	Compound Name	Source of Standard
Silica gel in plastic tray or Petri dish	Pentane extraction w/o derivatization	n-Heptacosane	Ant extraction
		13-Methylheptacosane	Ant extraction
		13, 15-Dimethylheptacosane	Ant extraction
		3-Methylheptacosane	Ant extraction
		3,9-Dimethylheptacosane	Ant extraction
Silica gel in bottles or vials	Pyridine extraction w/o derivatization	n-Heptacosane	Ant extraction
		13-Methylheptacosane	Ant extraction
		13, 15-Dimethylheptacosane	Ant extraction
		3-Methylheptacosane	Ant extraction
		3,9-Dimethylheptacosane	Ant extraction
		trans-2-Methyl-6-(*cis*-4-tridecenyl) piperidine	Ant extraction
		trans-2-Methyl-6-n-tridecylpiperidine	Ant extraction
		trans-2-Methyl-6-(*cis*-6-pentadecenyl) piperidine	Ant extraction
		trans-2-Methyl-6-n-pentadecylpiperidine	Ant extraction
Silica gel in bottles or vials	Water extraction with derivatization	Lactic acid	Sigma-Aldrich
		Malonic acid	Alfa Aesar
		Phosphoric acid	Sigma-Aldrich
		Glycerol	Sigma-Aldrich
Silica gel with excreta	No extraction with derivatization	Lactic acid	Sigma-Aldrich
		Alanine	Sigma-Aldrich
		Glycine	Sigma-Aldrich
		Malonic acid	Alfa Aesar
		Urea	Alfa Aesar
		Benzoic acid	Sigma-Aldrich
		N-acetylglycine	MP Biomedicals, Inc.
		Phosphoric acid	Sigma-Aldrich
		Glycerol	Sigma-Aldrich
		Succinic acid	MP Biomedicals, Inc
		Serine	Sigma-Aldrich
		Methylmaleic acid	Sigma-Aldrich
		Threonine	Sigma-Aldrich
		3-Methylglutaric acid	MP Biomedicals, Inc
		Malic acid	Sigma-Aldrich
		Aspartic acid	Sigma-Aldrich
		Glutamine	Sigma-Aldrich
		Phenylalanine	Sigma-Aldrich
		Suberic acid	Sigma-Aldrich
		Azelaic acid	Sigma-Aldrich
		Tyrosine	Sigma-Aldrich
		Hexadecanoic acid	Sigma-Aldrich
		2-Quinolinecarboxylic acid	Sigma-Aldrich
		Gluconic acid	Sigma-Aldrich
		Uric acid	MP Biomedicals, Inc
		Stearic acid	Sigma-Aldrich
		n-heptacosane	Ant extraction
		13-Methylheptacosane	Ant extraction
		13, 15-Dimethylheptacosane	Ant extraction
		3-Methylheptacosane	Ant extraction
		3,9-Dimethylheptacosane	Ant extraction

Discussion

Cuticular hydrocarbons and venom alkaloids were found in the nest materials, in agreement with the literature (Obin and Vander Meer, 1985; Vander Meer, unpublished data, cited Vander Meer and Lofgren, 1988; Storey et al., 1991). However, the results of this study show that red imported fire ants also deposited other compounds on nest materials.

Excreta were believed to be a source of element enrichment in mound soil of red imported fire ants (Herzog et al., 1976; Green et al., 1998, 1999), and organic compounds found in the excreta would possibly be accumulated in the mound. Although *S. invicta* is one of the most well-studied ant species in the United States, there is surprisingly little information on its excreta. Petralia et al. (1982) reported that larvae of *S. invicta* produced two forms of excretory products: a white precipitate composed of uric acid and a clear liquid of water and salts. Chen (2005) reported *S. invicta* excreted phosphoric acid. The chemistry of fire ant excreta has not been more fully examined until now.

Uric acid and urea were found in all brown or yellow silica gel samples, which suggested that the discoloration of the silica gel was due to the ant excreta. Urea excretion is not unusual in insects (Cochran, 1985), but it has never been reported as a nitrogenous excretory product of red imported fire ants. Many other nitrogenous compounds were also found in the colored silica gel, such as amino acids and 2-quinolinecarboxylic acid, raising interesting questions about the nitrogenous excretory mechanism of red imported fire ants, such as whether the amino acids and 2-quinolinecarboxylic acid also function as nitrogenous excretory products of *S. invicta*.

It was known that red imported fire ants excrete phosphoric acid (Chen, 2005). The results of this study show that fire ants also incorporate phosphoric acid into their nesting material because phosphoric acid was present in the water extractions of all samples collected inside the nests. Phosphoric acid and urea are well-known plant growth promoters. Thus, phosphoric acid and the nitrogen-containing excretory compounds may contribute to the abundant vegetation commonly found around fire ant mounds.

Glycerol was also found in the nesting materials and is known to be a humectant that promotes retention of moisture. Incorporation of glycerol into the nest material would influence the moisture balance inside the nest, and may contribute to microclimate regulation within the nest. This may be important when the environmental humidity is low, such as during a dry season.

Although all compounds identified inside the nest, such as lactic acid, malonic acid, phosphoric acid, and glycerol, also were found in excreta, the possibility of a secretory origin of these compounds cannot be excluded. Organic acids have been found as exocrine gland components of numerous ant species. A well-known example is formic acid, a component of poison gland secretions of formicine species (Hölldobler and Wilson, 1990). In addition to formic acid, acetic acid, butyric acid, isovalerianic acid, (D) 3-hydroxydecanoic acid, indoleacetic acid, and phenylacetic acid have been associated with ants of different species (Hölldobler and Wilson, 1990). Furthermore, Mikheyev (2003) reported four fatty acids and benzoic acid in the *S. invicta* male accessory glands, where they were believed to have a role in the formation of a mating plug. Benzoic acid was also found in the excreta of red imported fire ants. Because alates were present in all experiments, the possibility that benzoic acid might have come from the male accessory glands cannot be ruled out.

Incorporation of organic acids into nest material by red imported fire ants has not been reported. Organic acids are commonly recognized antimicrobial agents (Cherrington et al., 1991). For example, lactic acid, benzoic acid, and malic acid are used as food preservatives

for human consumption (http://www.lactose.co.uk/milkallergy/foodadditives200.html). The presence of these organic acids in the nesting materials may significantly affect the microorganism communities inside the nests, which in turn may influence colony fitness.

Knowledge of nest chemistry may contribute to our understanding of the behavior and chemical ecology of a nest-building ant species. The technique described herein may be useful in establishing profiles of ant-derived chemicals in the nests for other mound-building ant species.

Acknowledgments We thank Douglas A. Streett and Jianzhong Sun for critical reviews of an early version of the manuscript. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U. S. Department of Agriculture.

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J Chem Ecol (2007) 33:643–654
DOI 10.1007/s10886-007-9251-z

The Role of Vanadium in the Chemical Defense of the Solitary Tunicate, *Phallusia nigra*

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Received: 14 August 2006 / Revised: 26 December 2006 / Accepted: 29 December 2006 /
Published online: 31 January 2007
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Abstract Ascidians (sea squirts) may defend themselves from predators, biofouling competitors, and bacterial infection by producing secondary metabolites or sequestering acid, but many species also accumulate heavy metals, most notably vanadium. The defensive functions of heavy metals in ascidians remain unclear, and to this end, the solitary Caribbean tunicate, *Phallusia nigra*, was studied to localize vanadium in its tissues and to assess the defensive properties of vanadium-containing compounds. As determined by flame atomic absorption spectroscopy, the internal tissues and blood contained the highest vanadium concentrations (mean values of 2,280 and 1,886 ppm dry mass, respectively), followed by the tunic surface (871 ppm dry mass). Results of laboratory feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*, confirmed outcomes of past studies that demonstrated that vanadyl sulfate ($VOSO_4 \cdot 6H_2O$) and sodium vanadate (Na_3VO_4) were unpalatable to fish, although these salts do not accurately reflect the chelation environment or oxidation state of vanadium in living tunicates. Fresh preparations of whole tunic, internal tissues, and blood were unpalatable to fish, but freezing and thawing of internal tissues and blood rendered them palatable. Crude organic extracts of whole tunic and internal tissues contained vanadium metabolites (225 and 750 ppm dry mass, respectively) and were palatable to *T. bifasciatum*; crude extracts also exhibited no antimicrobial effects against a panel of four marine bacteria known to be pathogens of marine invertebrates (*Vibrio parahaemolyticus*, *Vibrio harveyi*, *Leucothrix mucor*, and *Deleya marina*). Nonacidic vanadium (+3) complexes neither deterred predation nor inhibited microbial growth, whereas acidic aqua vanadium (+3 and +4) complexes were unpalatable to *T. bifasciatum* and exhibited antimicrobial activity. Difficulties in decoupling low pH from oxidation state and chelation environment of vanadium prevent definitive conclusions about the importance of some vanadium metabolites, but low pH appears to be the principal agent of chemical defense for *P. nigra*.

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Keywords Tunicate · Ascidian · Predation · Chemical defense · Heavy metals · pH · Vanadium · Acid

Introduction

Ascidians are soft bodied, sessile fouling invertebrates that are common in benthic marine environments and are widely distributed from the tropics (Hernández-Zanuy and Carballo, 2001) to the poles (Sahade et al., 1998). They secrete a body covering, the tunic, which consists predominantly of polysaccharides and proteins. Calcareous spicules are also found in the tunic of some species (e.g., Lambert and Lambert, 1987). Spicules were once thought to provide a physical defense against predation; however, fish-feeding assays performed with calcareous spicules of tunicates (Lindquist et al., 1992; López-Legentil et al., 2006) have demonstrated that mineralized inclusions have no deterrent effect. Tissue toughness of the tunic and an overall low nutritional value of ascidians have also been thought to deter predation. Tarjuelo et al. (2002) found a positive correlation between palatability and energy content in six ascidian species, but other studies have failed to support this hypothesis (Lindquist et al., 1992).

Putative chemical defenses of tunicates include secondary metabolites and inorganic acids (Pawlik, 1993; Pisut and Pawlik, 2002; Tarjuelo et al., 2002; López-Legentil et al., 2006), but may also include heavy metals, most notably vanadium (Webb, 1939; Ciereszko et al., 1963; Swinehart et al., 1974; Stoecker, 1978, 1980b). Some marine organisms use low pH as a form of chemical defense, ascidians among them (Thompson, 1960). Some ascidians accumulate sulfuric acid (pH≤2.0) in bladder cells located within the tunic (Webb, 1939; Swinehart et al., 1974). Pisut and Pawlik (2002) demonstrated that food pellets with pH≤3.0 were deterrent in feeding assays with the bluehead wrasse, *Thalassoma bifasciatum*.

It has long been recognized that ascidians actively accumulate heavy metals, including manganese, magnesium, iron, molybdenum, niobium, tantalum, chromium, and titanium, but most commonly vanadium (Webb, 1939; Ciereszko et al., 1963). Elemental vanadium does not exist in nature, but rather in anionic and cationic form with oxidized states ranging from −1 to +5. The most common oxidation states of vanadium under physiological conditions occur as +3, +4, and +5 (Rehder, 1999). Vanadium has been identified in trace amounts in bacteria, plants, and animals, where it acts to facilitate oxidative and reductive pathways. The vanadium center is necessary for nitrogen-fixing bacteria as part of the vanadium–nitrogenase enzyme complex involved in the reduction of gaseous N_2 to NH_4^+.

The black sea squirt *Phallusia nigra* (= *Ascidia nigra*) is a large solitary phlebobranch ascidian that ranges from Florida to Texas and throughout the West Indies on pilings and sea walls. In the field, *P. nigra* does not show signs of predation, and the tunic is free of fouling organisms (Goodbody, 1962; Hirose et al., 2001). In comparison to other ascidians, *P. nigra* has some of the highest recorded vanadium concentrations and is one of the best studied with respect to vanadium chemistry. In *P. nigra*, vanadium is stored primarily as vanadium (+3)(~70%), but vanadium (+4) is also present (~30%; Webb, 1939; Swinehart et al., 1974; Kustin et al., 1976; Frank et al., 2003). The coordination chemistry of the metal has yet to be completely clarified (Dingley et al., 1982; Bruening et al., 1985). X-ray absorption spectroscopic (XAS) investigations of the blood cells of this tunicate suggest that vanadium resides in more than one ligand environment and includes both V (+3) and V (+4) complexed with water molecules and V (+3) chelated to

proteins containing catechol and pyrogallol moieties. These ligand environments include low molecular weight proteins derived from the amino acids 3,4-dihydroxyphenylalanine (DOPA) or 3,4,5-trihydroxyphenylalanine (TOPA) (Bruening et al., 1985; Frank et al., 2003).

The concentration of vanadium in seawater is between 0.0003 and 0.003 parts per million (ppm). Most marine organisms contain vanadium concentrations of 1 to 3 ppm dry mass, but some ascidians have been shown to accumulate vanadium to 3,000 ppm (Ciereszko et al., 1963; Swinehart et al., 1974). In seawater, vanadium exists as vanadate ($H_2VO_4^- / HVO_4^{-2}$), vanadium (+5). In *P. nigra*, vanadium (+5) is accumulated from seawater, reduced to V (+4) and then stored as V (+3) (Dingley et al., 1982; Brand et al., 1989; Michibata et al., 2003). Vanadium is sequestered primarily in blood cells called vanadocytes (Kustin et al., 1976; Brand et al., 1989; Hirose, 1999; Hirose et al., 2001; Frank et al., 2003), but can also be found in high concentrations in a thin layer located near the exterior tunic surface (Stoecker, 1978). The oxidation state of vanadium is influenced by both the pH of the localized environment and the chelating ligands (Rehder, 1999). The mechanism of vanadium uptake, reduction, transfer, and accumulation has yet to be clarified (Michibata et al., 2003).

Stoecker (1978, 1980a,b) proposed that heavy metals, particularly vanadium, provide ascidians with antipredatory and antifouling defenses. The importance of vanadium as an antipredatory chemical defense was examined by using feeding assays with fish and crabs incorporating the vanadium salts, vanadyl sulfate ($VOSO_4 \cdot 6H_2O$) and sodium vanadate ($NaVO_3$). These compounds had a minimum deterrent concentration of 1,250–3,333 ppm dry mass (~100 ppm wet tissue mass). However, vanadium in ascidian tissues occurs in biochemical complexes that differ from those found in salts used for feeding assays (e.g., Bruening et al., 1985; Frank et al., 2003).

Low pH is coupled with heavy metal accumulation and storage in adult ascidians. In an attempt to decouple the relationship between inorganic acids and vanadium, we compared two nonacidic vanadium (+3) complexes to two acidic aqua vanadium (+3 and +4) complexes in laboratory assays by testing for antipredatory and antimicrobial activity. For comparison, components of the vanadium complexes and vanadium salts were tested for antipredatory and antimicrobial activity. In addition, *P. nigra* tissues and blood samples, both fresh and previously frozen, and crude organic extracts of *P. nigra* whole tunic and internal tissues were also assayed. In this study, we addressed the following questions: (1) Does vanadium act as a chemical defense of *P. nigra*? (2) Are the deterrent properties of vanadium affected by complexation environment and associated pH?

Methods and Materials

Whole individuals of *P. nigra* were collected with substrate intact along sea walls and pilings in the Port Largo Canal, Key Largo, FL, USA, in October 2002 and January 2003. Individuals were collected by self-contained underwater breathing apparatus (SCUBA) at depths between 3 and 7 m. Substrate attached to the basal portion of tunicates was carefully removed and each individual was immediately weighed and a volume determined by submerging the animal in a graduated cylinder and recording the amount of seawater displaced.

Blood of *P. nigra* was isolated as described in Brand et al. (1989). In brief, blood was extracted by heart puncture with a sterile 3-ml syringe and an 18.5-gauge wide bore hypodermic needle. The blood collected from each individual was placed in a sterile 2-ml cryogenic vial. Extracted blood was flash frozen in an ethanol and dry ice slurry (~−70°C) to limit oxidative effects. The tunic of each individual was then dissected from the internal

tissues, and the volume and mass of each were determined. After dissection, both the tunic and internal tissues were rinsed with distilled water and frozen in separate containers. Specimens of *P. nigra* were collected for the purpose of obtaining samples of the tunic bladder cell layer. This layer was separated from the rest of the tunic by using a sterile scalpel (Stoecker, 1978; Hirose, 1999; Hirose et al., 2001). The volume and mass of the tunic bladder cell layer were recorded. After dissection, the bladder cells were flash frozen and stored as previously stated.

Tunic and internal tissues were chopped into small pieces and exhaustively extracted in a mixture of 1:1 dichloromethane is to methanol (DCM:MeOH) followed by 100% MeOH using previously described methods (Pawlik et al., 1995). Frozen samples of tunic, tunic bladder cells, internal tissues, and blood were freeze dried until a constant mass was attained. Subsamples of whole tunic, internal tissues, tunic bladder cell layer, blood samples, and crude extracts of tunic and internal tissues were digested in hot concentrated 2:1 nitric acid is to sulfuric acid (HNO_3:H_2SO_4) (Sigma-Aldrich, trace metal grade) in a 25-ml Teflon digestion vessel. The concentration of vanadium from each sample was measured with flame AAS by using the standard curve method with a Perkin Elmer 3110 AAS and nitrous oxide (N_2O_2) (Kustin et al., 1976; Stoecker, 1978).

A solution of vanadium chloride (VCl_3, Fisher Scientific) in 1 M HCl was used to simulate the hexaaqua V (+3) component of *P. nigra* blood cell chemistry. The V (+3) fraction of the blood chelated by DOPA or TOPA-like ligand was simulated by V (acetylacetonate)$_3$ (Strem Chemicals Inc.) and K_3[V(catecholate)$_3$]·1.5 H_20 (Cooper et al., 1982), which was prepared as needed. The V (+4) fraction of the blood cells was reproduced as $VOSO_4$·6 H_2O in 1 M HCl (Frank et al., 2003). The vanadium (+3) tris (catecholate) complex, K_3[V(catecholate)$_3$] (molecular weight=519.55), was synthesized by using the methods described by Cooper et al. (1982). All operations were carried out with standard Schlenk techniques under nitrogen (N_2) atmosphere. Vanadium (+4) catecholate and vanadium (+3) catecholate complexes can be distinguished from one another by the presence of a V=O stretch at 977 cm^{-1} within the infrared (IR) spectra of V (+4) tris(catecholate) and the absence of this band in corresponding V (+3) tris(catecholate) complexes (Selbin, 1966). The techniques of infrared spectroscopy with a potassium bromide pellet (KBr) and ultraviolet (UV) spectroscopy were used to confirm the presence and coordination of vanadium in all synthesized vanadium compounds. Reaction products were purified by recrystallization.

Stock solutions of each vanadium-containing compound were prepared on the basis of the molecular weight of the vanadium fraction in proportion to the molecular weight of the entire vanadium complex. Three stock solutions of each vanadium complex with vanadium concentrations of 1,000, 2,000, and 4,000 μg/ml were prepared relative to known vanadium concentrations in tissues of *P. nigra*. The pH of each stock solution, and of ascidian blood, was determined by pipetting 30 μl of solution onto analytical pH strips (EM-reagents 9580, 9581, and 9590). The pH of fresh and frozen tissues was also determined by using analytical pH strips by pressing the test strip to the surface of the tissue with light pressure. Each stock solution and tissue type was measured three times to determine a mean pH value; the pH values were consistent and did not vary with each reading.

Fish feeding assays were performed with the bluehead wrasse, *T. bifasciatum*, a generalist Caribbean reef predator, as described in Pawlik et al. (1995). Vanadium complexes, reaction precursors, tissue and blood extracts were incorporated into a food matrix composed of freeze-dried squid mantle (5 g), alginic acid (Sigma-Aldrich, Na-salt form) (3 g), and distilled water (100 ml) (see Pisut and Pawlik, 2002 for discussion of food value of ascidian tissues). The mixture was stirred until homogenous and then packed into a

3.0-ml syringe and extruded into a 0.25-M calcium chloride ($CaCl_2$) solution, which caused the squid matrix to harden. After a few minutes, the spaghetti-like strand was removed from the $CaCl_2$ solution and rinsed with deionized water. The resulting string was cut with a razor blade into 4.0-mm long pellets. Control pellets were made in the same manner with the addition of the same solvent as used to dissolve the metabolite or extract for the corresponding treatment. Some highly basic compounds reacted with the squid and alginic acid matrix and caused it to harden before being extruded into the $CaCl_2$ solution. These compounds were instead incorporated into a food matrix composed of freeze-dried squid mantle (5 g), carageenan (3 g) (Sigma-Aldrich, type 1 commercial grade), and distilled water (100 ml). The mixture was stirred until homogenous and heated in a microwave until boiling. The mixture was then quickly remixed and poured onto a clean bench top and allowed to harden. Once the carageenan mixture had cooled, the resulting gel was cut with a razor blade into 4 mm long pellets. Control pellets were made in the same manner with the addition of the same solvent as used to dissolve the metabolite or extract for the corresponding treatment.

Five 30 gallon aquaria were divided into 24 cells, and groups of two to four yellow-phase *T. bifasciatum* were placed in each cell. Groups of fish were randomly chosen during feeding assays and offered a control food pellet, followed by a treatment pellet. If the fish rejected the treatment pellet, another control pellet was offered to determine whether the fish had stopped feeding; groups of fish that would not eat control pellets were not used in assays. A pellet was considered rejected if not eaten after a minimum of three attempts by one or more fish to take it into their mouth cavity or if the pellet was approached and ignored after one such attempt. Fish were fed 10–30 min before assays to limit effects due to extreme hunger. Uneaten pellets were removed from tanks after each assay. By using the Fisher exact test, a significant difference in the number of treated and control pellets eaten was determined for an individual 10-replicate assay if 4 or more pellets were rejected ($P \leq 0.043$, one-tailed test). Therefore, treatments were considered deterrent if the mean number of pellets eaten was ≤ 6 (Pawlik et al., 1995).

To assess the effect of basic (high) pH on palatability, food pellets were made as before with carageenan and the addition of 1 N sodium hydroxide (NaOH) that provided the desired pH, as indicated by pressing pellets against analytical test strips. Control pellets were made in the same manner, but did not include the addition of base. The mixture was stirred and microwaved for 30 sec. The heated mixture was poured onto a clean bench top and allowed to harden. Food pellets were cut from the hardened gel. Feeding assays were performed as before; however, the pH of food pellets was determined with analytical test strips both before assay and after pellets were rejected to assure consistency in pellet pH values. The pH of successive assays was increased within a range starting at pH 7.0 to 14.0. Control pellets had a pH of 7.0.

Antimicrobial activity was assessed for vanadium-containing compounds and reaction precursors and crude organic extracts of *P. nigra* tunic and internal tissues. Each compound and tissue extract was tested against an assembly of four marine bacteria that included: *Vibrio harveyi* (Microbial Identification, Inc., MIDI, Newark, DE, USA), *Deleya marina* (American Type Culture Collection (ATCC) 35142), *Vibrio parahaemolyticus* (ATCC 27969), and *Leucothrix mucor* (ATCC 25906). The bacterium *V. harveyi* was isolated and identified as part of a previous study by Kelly et al. (2003) and has been recognized as an opportunistic pathogen of certain fish and marine invertebrates. The marine microbe *D. marina* is a common surface fouling bacterium, and both *V. parahaemolyticus* and *L. mucor* are known marine pathogens that can cause disease in marine invertebrates (Kelly et al., 2003). Standard disc-diffusion antimicrobial assays were performed by inoculating each

bacterial test strain onto the surface of a YP agar medium plate: peptone (1.0 g l^{-1}) and yeast extract (1.0 g l^{-1}) in filtered seawater (32–36‰) with the addition of agar (15 g l^{-1}) (Newbold et al., 1999). Culture concentrations were measured before each antimicrobial bioassay by using a Milton Roy Spectronic 20 D at 500 nm. Only cultures that had an absorbance between 0.2 and 0.5 were used to inoculate bioassay media plates. A 100-µl aliquot of inoculated broth was spread with a sterile glass rod over the entire agar surface to form an evenly distributed film. Compounds were dissolved in solvent and tested by pipetting 30 µl onto a sterile circular paper disc (Becton Dickinson, 6×0.9 mm, disc volume=30 µl) and allowing the solvent to evaporate. Crude organic extracts were dissolved in methanol before transfer to paper disc. All solvents used to dissolve air-sensitive materials were degassed of oxygen before use, and all stock solutions were stored under inert atmosphere with purified nitrogen. After pipetting the stock solution onto a paper disc, the solvent was evaporated under nitrogen. Stock solutions were monitored for color change that would indicate oxidation events. If oxidation occurred, the solutions were remade before use in the assay. The discs were placed on the surface of the inoculated agar (up to nine per plate). By pressing lightly on the disc with an analytical pH strip, the pH of each disc was checked once the discs had rehydrated after being placed on the agar plate. All strains were incubated at room temperature (22°C), and a confluent film developed over the entire agar surface after 24 hr. Clear halos (zones) surrounding the discs denoted areas of inhibited microbial growth. The computer-imaging program Image J (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/) was used to calculate area of inhibition zones by subtracting the area of the disc (28.27 mm^2) from the total area of clear zone. Compounds that exhibited inhibition zones (total area of clear zone minus the area of the disc) of greater than or equal to 50 mm^2 were considered to possess acute antimicrobial activity. Statistical analyses were not used to assign significance of differences between treatments because the disc diffusion assay is used as a qualitative measure of antibacterial activity (Newbold et al., 1999).

Results

Mean vanadium concentrations in tissues, blood samples, and crude organic extracts of *P. nigra* ranged from 2,280 ppm dry mass (63 ppm wet mass) in internal tissues to 225 ppm dry mass (7 ppm wet mass) in crude organic extracts of the ascidian tunic (Table 1). Assay foods prepared from tissue and blood of *P. nigra* were variable in their palatability to the

Table 1 Flame atomic absorbance spectroscopy results of the vanadium content (ppm ± SD) of freeze dried *P. nigra* tissues, blood samples, and crude organic extracts of whole tunic and internal tissues

Sample	*N*	Vanadium Content (ppm)	
		Dry Mass	Wet Mass
Whole tunic	6	259±157[a]	5±3
Tunic bladder cell layer	7	871±390[b]	18±8
Internal tissues	5	2,280±822[c]	63±23
Blood	7	1,886±567[c]	38±12
Crude extract, internal tissues	4	750±289	31±12
Crude extract, tunic	4	225±126	7±4

Wet mass concentrations were converted from dry mass. Significant differences in concentrations for tissues and blood are shown as different superscript letters (one-way ANOVA, N=3, F=41.02; Ryans–Einot–Gabriel–Welsch Multiple Range Test, homogeneous subsets at P=0.05).

generalist consumer fish, *T. bifasciatum*, with fresh material highly deterrent, while frozen material was readily eaten (Fig. 1). Both freshly dissected whole tunic tissue (pH ~ 2.0) and previously frozen tunic (pH=7) were unpalatable to *T. bifasciatum*. Freezing and thawing neutralized low pH of tissues but did not affect the vanadium content. In all cases, fishes

Treatment	Vanadium Concentration µg/ml	Food Pellet pH
Whole Tunic (fresh)	5 ± 3	2.0
Whole Tunic (frozen)	5 ± 3	7.0
Soft Body (fresh)	63 ± 23	7.0
Soft Body (frozen)	63 ± 23	7.0
Blood (fresh)	38 ± 12	2.0
Blood (frozen)	38 ± 12	7.0
Crude Extract: Tunic	7 ± 4	5.0
Crude Extract: Soft Body	31 ± 12	5.0
$V(acetylacetonate)_3$	1000	6.5
$V(acetylacetonate)_3$	2000	6.5
$V(acetylacetonate)_3$	4000	6.5
$K_3[V(catecholate)_3]$	1000	11.0
$K_3[V(catecholate)_3]$	2000	11.0
$K_3[V(catecholate)_3]$	4000	11.0
VCl_3 in 1M HCl	1000	0.0
VCl_3 in 1M HCl	2000	0.0
VCl_3 in 1M HCl	4000	0.0
$VOSO_4$ in 1M HCl	1000	0.0
$VOSO_4$ in 1M HCl	2000	0.0
$VOSO_4$ in 1M HCl	4000	0.0
$VOSO_4$	1000	2.2
$VOSO_4$	2000	1.9
$VOSO_4$	4000	1.6
Na_3VO_4	1000	11.0
Na_3VO_4	2000	11.0
Na_3VO_4	4000	11.0

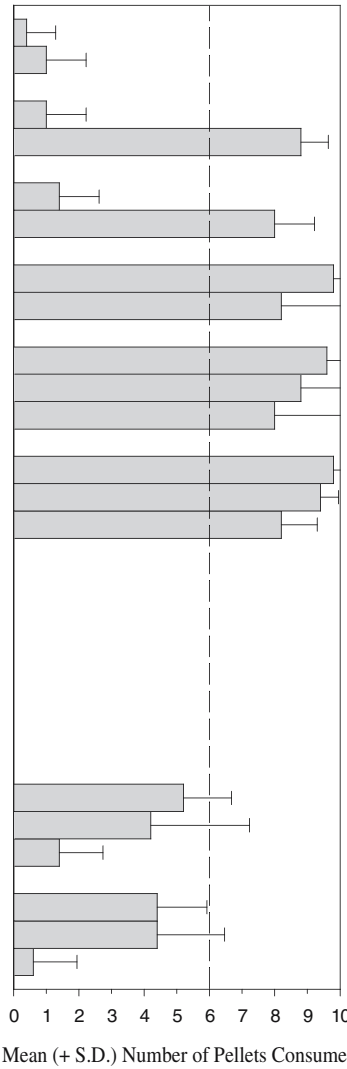


Fig. 1 Consumption by the bluehead wrasse, *T. bifasciatum*, of food pellets treated with tissue and blood of *P. nigra*, vanadium complexes, and vanadium salts. Compounds were considered deterrent if the mean number of pellets eaten for any individual assay were less than or equal to 6 ($P \leq 0.043$, Fisher exact test, one-tailed), as indicated by the *dotted line* on the graph. Mean + SD shown for five replicate assays of each treatment. Vanadium concentration and pH of food pellet are shown for each treatment

approached and mouthed the pieces of the tunic but did not eat them. Food pellets made with finely chopped tunic tissue, fresh and frozen, were also mouthed and the squid matrix consumed; however, the bulk of the tunic tissue was rejected. Freshly dissected soft body internal tissues (pH=7) were unpalatable to *T. bifasciatum*, whereas frozen and thawed tissues were consumed, but in this case, there was no change in pH after freezing and thawing. Fishes rejected freshly collected *P. nigra* blood (pH ~ 2) incorporated into food pellets, whereas frozen and thawed blood (pH=7) was palatable.

In the same feeding assays with *T. bifasciatum*, crude organic extracts of ascidian tissues, vanadium complexes, and vanadium salts were also variably deterrent, with the greatest level of unpalatability associated with low pH (Fig. 1). Crude organic extracts of internal tissues contained high vanadium concentrations (750 ppm dry mass=31 ppm wet mass, Table 1), but were palatable to *T. bifasciatum* (Fig. 1). The nonacidic vanadium (+3) compounds bound to DOPA/TOPA-like chelation environments were also tested, and V (acetylacetonate)$_3$ and K_3[V(catecholate)$_3$] were palatable to *T. bifasciatum* at all of the concentrations assayed. Food pellets with vanadyl sulfate were unpalatable to *T. bifasciatum*, but at the highest pH assayed (pH=2.2), a mean of five pellets were eaten, with decreasing palatability as the vanadium content was increased and the pH decreased. Food pellets made with the vanadium salt sodium vanadate at all concentrations (pH=11) were unpalatable to *T. bifasciatum* (Fig. 1). This effect was not attributable to high pH because food pellets treated with NaOH to generate high pH were only unpalatable to *T. bifasciatum* at pH≥13.0 (Fig. 2). The pH of food pellets treated with NaOH was tested after rejection by assay fishes and had not changed.

By using a standard disc-diffusion assay, crude organic extracts of tunic and internal tissues, vanadium complexes, and vanadium salts were assessed for antimicrobial activity against a panel of four marine bacteria known to infect marine invertebrates. Crude organic extracts of dissected tunic and internal tissues (N=3 for each tissue type vs. each bacteria) of four individual tunicates did not exhibit any antimicrobial activity against the panel of marine bacterial lines tested (data not shown). The nonacidic vanadium (+3) complexes, V (acetylacetonate)$_3$, and K_3[V(catecholate)$_3$] did not exhibit any antimicrobial activity against the entire panel of marine bacteria; however, the acidic aqua vanadium (+3 and +4) complexes inhibited microbial growth (Table 2). Although assayed at equivalent vanadium concentrations, the two vanadium salts had different effects on the panel of bacteria.

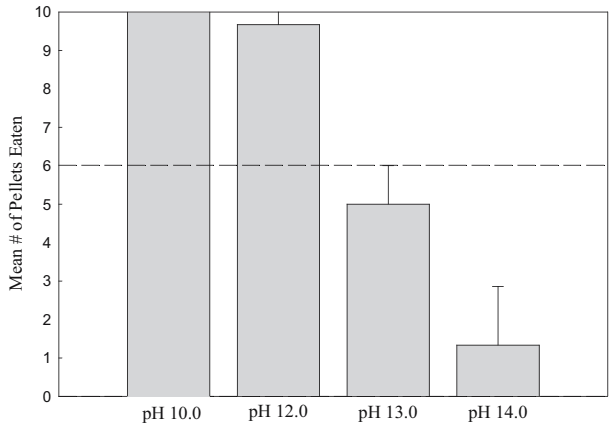


Fig. 2 Consumption by *Thalassoma bifasciatum* of food pellets treated with sodium hydroxide. Fish consumed ten control pellets in all cases. Treatments were considered deterrent if the number of pellets eaten was less than or equal to 6 (P≤0.043, Fisher exact test, one-tailed), as indicated by the dotted line on the graph. Mean + SD shown for three replicate assays of each treatment

Sodium vanadate at all concentrations (pH=11) did not inhibit microbial growth, whereas vanadyl sulfate (pH 1.9 to 3.6) did produce clear zones of inhibition (Table 2)

Discussion

The presence of low pH and heavy metals in the tissues of ascidians has long been recognized. Stoecker (1978) first hypothesized that inorganic acids and heavy metals, most notably vanadium, provided ascidians with effective antipredatory and antifouling defenses. Parry (1984) disputed Stoecker's results, but arguments by Parry (1984) were correlative and based on the existence of other tunicate species in similar habitats that contained neither vanadium nor acid, yet remained uneaten or fouled. Parry (1984) did not consider the presence of secondary metabolites as defenses that might function in the absence of vanadium and sulfuric acid. Stoecker (1980a) conducted feeding assays with fish and crabs with the salts vanadyl sulfate and sodium vanadate. These salts putatively represented the oxidation state of vanadium when it was released from the ascidian during a predation event. The results of the feeding assays reported herein that used the bluehead wrasse, *T. bifasciatum*,

Table 2 Antimicrobial activity [mean area of inhibition (standard deviation), mm^2] of vanadium compounds and vanadium salts vs. bacterial test strains using standard disc diffusion assay

Compound	Vanadium Concentration (μg/ml)	Solution pH	Disc pH	*Vibrio parahaemolyticus* Mean Area of Inhibition	*Deleya marina* Mean Area of Inhibition	*Leucothrix mucor* Mean Area of Inhibition	*Vibrio harveyi* Mean Area of Inhibition
V (acetylacetonate)$_3$	4,000	7.0	4.0	0	0	0	0
V (acetylacetonate)$_3$	2,000	7.0	5.0	0	0	0	0
V (acetylacetonate)$_3$	1,000	7.0	6.0	0	0	0	0
K3[V(catechol)$_3$]	4,000	11.0	10.0	0	0	0	0
K$_3$[V(catechol)$_3$]	2,000	11.0	10.0	0	0	0	0
K$_3$[V(catechol)$_3$]	1,000	11.0	9.0	0	0	0	0
VCl_3 in 1 M HCl	4,000	0.0	2.5	+++	+++	+++	+++
VCl_3 in 1 M HCl	2,000	0.0	3.3	+	+	+	+
VCl_3 in 1 M HCl	1,000	0.0	3.6	+	+	+	+
$VOSO_4$ in 1 M HCl	4,000	0.0	1.6	+	+++	+++	+++
$VOSO_4$ in 1 M HCl	2,000	0.0	1.9	+	+	+	+
$VOSO_4$ in 1 M HCl	1,000	0.0	2.2	+	+	+	+
$VOSO_4$	4,000	1.6	1.9	+	+++	+++	+++
$VOSO_4$	2,000	1.9	2.5	+	+	+	+
$VOSO_4$	1,000	2.2	3.6	+	+	+	+
Na_3VO_4	4,000	11.0	10.0	0	0	0	0
Na_3VO_4	2,000	11.0	9.0	0	0	0	0
Na_3VO_4	1,000	11.0	8.0	0	0	0	0

0 No activity, +clear zone of inhibition present, +++clear zone of inhibition greater than or equal to 50 mm^2

corroborate those of Stoecker (1980a) that used the fishes *Fundulus heteroclitus* (common killifish), *Abudefduf saxatilis* (sergeant major), and *Haemulon sciurus* (blue striped grunt). However, salts of vanadium do not accurately reflect vanadium as it is found in the tissues and blood of *P. nigra*. Furthermore, pH and vanadium accumulation and storage in adult ascidians are coupled, making it difficult to determine the chemical deterrence of the individual components.

This study is the first to attempt to decouple the chemical deterrence of vanadium from low pH in assessing the chemical defense of an ascidian. The presence of vanadium did not always deter predation or inhibit microbial growth (Fig. 1, Table 2), whereas low pH consistently did both. Whole tunic deterred feeding despite having a low concentration of vanadium, regardless of whether it was assayed at pH 2 or 7 (Fig. 1), suggesting a physical defense of the outer body covering. Crude extracts of tunic and internal tissues that contained vanadium but were not acidic neither deterred predation nor inhibited microbial growth. Vanadium species decoupled from acidity by chelation to DOPA/TOPA-type ligands did not deter predation or inhibit microbial growth; however, acidic aqua vanadium (+3 and +4) complexes were deterrent in both assay systems.

Localization of chemical defenses to specific regions of an organism is exhibited in terrestrial plants and marine organisms (Zangerl and Rutledge, 1996; Dworjanyn et al., 1999; Van Alstyne et al., 1999) and has also been suggested in tunicates (Pisut and Pawlik, 2002; Tarjuelo et al., 2002). Surface associated defenses may enhance the survival of an organism by providing a mechanism to protect against predation, deter fouling organisms, and control epibiont populations (Wahl et al., 1994). Acidity is important in deterring potential predators and is probably effective against most grazers and browsers (Thompson, 1960; Stoecker, 1980a). Davis and Wright (1989) hypothesized that surface acidity would be neutralized by the buffering capacity of seawater, but Pisut and Pawlik (2002) demonstrated that low pH persists in tissues for some time. Brief localized exposure to low pH may be enough to deter a potential predator (Hirose et al., 2001). Pisut and Pawlik (2002) demonstrated that pieces of the acidic tunic of *Ascidia interrupta* were unpalatable to *T. bifasciatum* in feeding assays, whereas crude organic extracts of the tunic were palatable. Moreover, epibionts are rare on species that have acidic tunics (Stoecker, 1980a).

In the solitary tunicate *P. nigra*, low pH and vanadium are associated with the tunic exterior. Acidic fluid is released from bladder cells localized near the exterior surface when the tunic is bruised or damaged (Hirose et al., 2001; Pisut and Pawlik, 2002). Vanadium is not evenly distributed throughout *P. nigra* tissues (Table 1), corroborating the previous work of Stoecker (1978) and Ciereszko et al. (1963). Vanadium is stored mainly in blood cells called vanadocytes, but is also accumulated at the tunic surface (Kustin et al., 1976; Brand et al., 1989; Hirose, 1999; Hirose et al., 2001; Frank et al., 2003). Interestingly, vanadocytes are able to migrate from blood vessels located in the tunic and accumulate in the pigmented layer located at the tunic surface. However, the coordination environment of vanadium at the tunic surface has yet to be fully clarified.

Acidic tissues are repellant to most fish (Thompson, 1960) and may primarily explain why *P. nigra* is unpalatable to fish predators. Tissues associated with low pH were unpalatable to *T. bifasciatum*; however, these same tissues that were frozen and then thawed exhibited higher pH values and were more palatable, perhaps, because of the buffering capacity of spicules or salts in the tunic. Although this process would not affect the presence of vanadium in these tissues, the oxidation state and ligand coordination associated with vanadium could be affected due to changes in the storage environment and oxidative effects of cell lysis. Non-chelated vanadium (+3) ions are unstable in air or moisture and are rapidly oxidized at pH≥2 (see review in Rehder, 1999). Interestingly, tunic

tissue pieces from both fresh and frozen *P. nigra* were both unpalatable to *T. bifasciatum* despite differences in pH, a result that cannot be readily explained.

In standard disc diffusion assays, crude organic extracts of the tunic and internal tissues of *P. nigra* did not inhibit the growth of any of the bacteria assayed. Nonacidic vanadium (+3) compounds also did not inhibit microbial growth, whereas low pH and acidic aqua vanadium (+3 and +4) complexes did. Although assayed at equivalent vanadium concentrations, the two vanadium salts had different effects on the panel of bacteria. Sodium vanadate (pH=11) did not inhibit microbial growth, whereas vanadyl sulfate (pH 1.9 to 3.6) did produce clear zones of inhibition. Acidic fluids that are released from ruptured bladder cells may be able to kill or inactivate bacteria or other microorganisms that would otherwise gain access to tunic tissues. Difficulties in decoupling low pH from oxidation state and chelation environment of vanadium prevent definitive conclusions about the defensive properties of some vanadium metabolites, but low pH appears to be the principal agent of chemical defense for *P. nigra*.

Acknowledgments This research was funded by grants from the NOAA/NURC Program (NOAA-NA96RU-0260), from the National Science Foundation Biological Oceanography Program (OCE-0095724, 0550468), and by a GlaxoWellcome Ocean and Human Health Fellowship. We thank Robert D. Hancock, Richard M. Dillaman, Richard D. Lancaster, Stephen A. Skrabal, and S. Bart Jones for advice and technical support.

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J Chem Ecol (2007) 33:655–668
DOI 10.1007/s10886-006-9245-2

Jasmonic Acid-Induced Changes in *Brassica oleracea* Affect Oviposition Preference of Two Specialist Herbivores

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Received: 6 September 2006 / Accepted: 18 December 2006 /
Published online: 2 March 2007
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Abstract Jasmonic acid (JA) is a key hormone involved in plant defense responses. The effect of JA treatment of cabbage plants on their acceptability for oviposition by two species of cabbage white butterflies, *Pieris rapae* and *P. brassicae*, was investigated. Both butterfly species laid fewer eggs on leaves of JA-treated plants compared to control plants. We show that this is due to processes in the plant after JA treatment rather than an effect of JA itself. The oviposition preference for control plants is adaptive, as development time from larval hatch until pupation of *P. rapae* caterpillars was longer on JA-treated plants. Total glucosinolate content in leaf surface extracts was similar for control and treated plants; however, two of the five glucosinolates were present in lower amounts in leaf surface extracts of JA-treated plants. When the butterflies were offered a choice between the purified glucosinolate fraction isolated from leaf surface extracts of JA-treated plants and that from control plants, they did not discriminate. Changes in leaf surface glucosinolate profile, therefore, do not seem to explain the change in oviposition preference of the butterflies after JA treatment, suggesting that as yet unknown infochemicals are involved.

Keywords Host plant selection · Glucosinolates · *Pieris rapae* · *Pieris brassicae* · Cabbage · Performance · Octadecanoid pathway

Introduction

Plants can be attacked by many herbivorous insects and have evolved a variety of defense strategies, including morphological barriers, synthesis of toxic or repellent secondary metabolites, and the release of synomones that attract natural enemies of the herbivores.

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These defenses can be constitutive, i.e., expressed independent of the presence of an attacker, or inducible, in which case defense compounds accumulate in response to attack (Karban and Baldwin, 1997). Herbivores can detect induced defensive compounds and respond by avoiding these plants, which signal lower suitability as a host plant (Landolt, 1993; De Moraes et al., 2001; Kessler and Baldwin, 2001; Meiners et al., 2005). Induced plant defense can affect herbivorous insects directly through the production of toxic compounds or indirectly through the production of cues that indicate intra- or interspecific competition for the herbivores (Schoonhoven et al., 2005). Moreover, induced plant defense signals can reduce the enemy-free space for the herbivores. For parasitoids and predators, induced infochemicals may indicate the presence of their host or prey on the plant (Turlings et al., 1990; Dicke and Vet, 1999). Phenotypic changes in individual plants may therefore affect insects at different trophic levels, and thus, the composition of the insect community and food web associated with the plant (Price et al., 1980; Van Zandt and Agrawal, 2004; Takabayashi et al., 2006).

Already in the 19th century, Kirby and Spence (1863) observed that *Pieris brassicae* females preferred to lay their eggs on plants devoid of eggs. Later, this was confirmed under more controlled conditions by Rothschild and Schoonhoven (1977) for both *P. brassicae* and *Pieris rapae*. This avoidance of infested plants is caused by a physiological response of the plant to oviposition, rather than by compounds excreted by the butterflies themselves (Blaakmeer et al., 1994). The butterflies also avoid egg deposition on leaves with feeding larvae (Rothschild and Schoonhoven, 1977). It was postulated that butterflies avoid laying eggs on herbivore-infested plants because herbivore attack induces defense compounds in plants that can influence the performance of their offspring and to reduce the risk of inter- or intraspecific competition and parasitism (Thompson and Pellmyr, 1991; Shiojiri et al., 2002). Egg-induced chemical changes in *Brassica* plants are also known to arrest *Trichogramma* parasitoids that parasitize *Pieris* eggs (Fatouros et al., 2005).

Oviposition-site selection involves an important behavioral decision in the life cycle of an herbivorous insect because hatching larvae have limited dispersal capacity (Renwick and Chew, 1994). *P. rapae* is a solitary butterfly that lays one egg at a time, whereas *P. brassicae* is gregarious and lays batches of about 20–100 eggs. *P. rapae* appears to spread the risk of larval mortality, laying few eggs within any patch. This has the advantage of being able to exploit isolated plants (Davies and Gilbert, 1985). *P. brassicae*, however, needs patches of plants because one large egg batch will require more than one plant for all caterpillars to develop into adults. Oviposition-site selection is performed in consecutive phases of searching and contact evaluation. *P. rapae* butterflies use visual, olfactory and tactile cues during these phases (Rothschild and Schoonhoven, 1977; Renwick and Radke, 1988). Acceptance of a site may be determined by the balance of positive and negative factors (Renwick and Radke, 1988). Renwick and Radke (1988) suggest that olfaction does not play a role in attraction to a host plant, but may be involved in avoidance of non-host plants. *P. rapae* and *P. brassicae* are crucifer specialists and are known to use glucosinolates, toxic secondary metabolites characteristic for Brassicaceae, as oviposition stimulants. Glucobrassicin and sinigrin are effective oviposition stimulants for *P. brassicae* and *P. rapae* (Renwick et al., 1992; Van Loon et al., 1992a).

A major signal-transduction pathway involved in induced plant defense is the octadecanoid pathway (Arimura et al., 2005). A central compound in the pathway is jasmonic acid (JA), which has an important role in direct and indirect defense in many plant species. In response to JA or methyl jasmonate (MeJA) treatment, increased concentrations of several compounds have been documented in a range of plant species, e.g., proteinase inhibitors (Moura and Ryan, 2001), polyphenol oxidases (Thaler et al., 1996), nicotine

(Baldwin et al., 1996), trypsin inhibitors (Cipollini and Sipe, 2001), glucosinolates (Cipollini and Sipe, 2001; Van Dam et al., 2004; Mewis et al., 2005), and increased volatile emission (Boland et al., 1995; Dicke et al., 1999; Koch et al., 1999).

Herbivores are reported to be affected by JA treatment of plants. Several studies have focused on the larval stage of the herbivores and have shown reduced relative growth rates and leaf consumption (Van Dam et al., 2000; Gols et al., 2003; Van Dam et al., 2004). In field experiments, spraying of JA decreased the abundance of caterpillars, flea beetles, aphids, and thrips (Thaler et al., 2001). Other studies addressed the influence of JA application to plants on oviposition-site selection behavior of adult herbivores. These studies showed that JA application can result in induced resistance as well as induced susceptibility (Stanjek et al., 1997; Kessler and Baldwin, 2001; Lu et al., 2004).

Here, we studied how JA application affects oviposition of two specialist herbivores on cabbage, *Pieris rapae* L. and *P. brassicae* L. (Lepidoptera: Pieridae) that are closely related, yet differ drastically in the amount of eggs they put on one plant. Our study is the first to compare closely related herbivores with a different oviposition strategy, which might affect the consequences of JA-induced responses. JA is known to mediate the induction of chemical defense responses in plants to feeding damage and deposition of eggs (Dicke and Van Poecke, 2002; Hilker and Meiners, 2006; Mumm and Hilker, 2006). By using JA, we were able to examine the effects of induced defense responses in cabbage. Moreover, JA application has the advantage that visually detectable damage and the presence of herbivores or eggs are avoided. Finally, JA allows control over the strength of induction through controlled dosages. We hypothesized that JA treatment would inhibit the oviposition of the butterflies. We made solvent extracts to address the identity of the active plant compounds that influenced butterfly behavior. Rather than testing whole-leaf extracts, however, we extracted the glucosinolates from the surface of both control and JA-treated plants and tested the oviposition preference of the butterflies for these glucosinolate fractions on a neutral substrate. Furthermore, we included a control experiment to exclude a potential direct effect of JA on oviposition behavior. We addressed the following questions: (1) does JA treatment of cabbage plants affect host plant selection of the two *Pieris* butterfly species; (2) are there differences between solitary and gregarious butterflies; (3) does JA treatment affect glucosinolate levels in leaf surface extracts; and (4) do changes in glucosinolate levels determine the changes in oviposition preference?

Methods and Materials

Plants and Insects Brussels sprouts plants, *Brassica oleracea* var. *gemmifera* L. (Brassicaceae) cultivar Cyrus were grown from seed in a greenhouse in plastic pots (11×11×11 cm) at 20–28°C, 40–80% RH and a 16-hr light/8-hr dark photoperiod. All experiments were conducted with 6- to 7-wk-old plants. Stock colonies of the large cabbage white *P. brassicae* and the small cabbage white *P. rapae* were maintained on Brussels sprouts in a climatized room at 20–22°C, 50–70% RH and a 16-hr light/8-hr dark photoperiod.

Chemical Analysis Brussels sprouts plants were sprayed with 0.1 mM JA or control solution. JA ((±) jasmonic acid, purity >97%; Sigma-Aldrich, St Louis, MO, USA) was applied to the surface of the leaves, i.e., plants were sprayed with a JA solution with 0.1% Tween 20 until run-off or just with 0.1% Tween 20 for the control. The next day, glucosinolates (GLS) were extracted from the surface of the intact Brussels sprouts leaves. Each sample consisted of four leaves (between the third to sixth leaf from the base of a

plant) that were cut at the base of the petiole. Directly after cutting, the lamina was dipped for 5 sec in 300 ml of dichloromethane, and after a 5-sec interval, it was dipped for 5 sec in 150 ml of methanol (Städler and Roessingh, 1990; Van Loon et al., 1992a; Griffiths et al., 2001). The methanol was evaporated from the crude methanol dip-volume with a rotary evaporator (IKA-Werke GmbH, Staufen, Germany). For each treatment, 11 plants were sampled. The extract was redissolved in methanol, desulphatased on a DEAE-Sephadex A25 column, and separated on a reverse phase C-18 column by using high performance liquid chromatography (HPLC) as described in Van Dam et al. (2004). Glucosinolate detection was performed with a photodiode array (PDA) detector (200–350 nm) with 229 nm as the integration wavelength. Sinigrin (sinigrin monohydrate, ACROS, NJ, USA) was used as an external standard. We used the correction factors at 229 nm from Buchner (1987) and the EC (EC, 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, MPI Chemical Ecology and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). The surface area was measured directly after dipping, and the dry mass of the leaves was measured after drying at 50°C for 72 hr. The GLS content was calculated in pmol per cm^2 leaf material.

Herbivore Oviposition Preference Test Pieris adults emerged from pupae in a large oviposition cage (67×100×75 cm) in a greenhouse compartment at 22–24°C and 50–70% RH. Apart from natural daylight, cages were illuminated by sodium vapor lamps (type SON-T, 500 W, Philips, The Netherlands) from 8:00 A.M. until 2:00 P.M. In this cage, they were provided with a 10% sucrose solution and an oviposition substrate; depending on the experiment, a plant or an artificial leaf made of green cardboard paper sprayed with sinigrin. For the experiments, one male and one female butterfly were introduced per oviposition cage (67×50×75 cm) in the same greenhouse compartment, on the day before the experiment. In these cages, the butterflies were also provided with sucrose solution. At 8:30 A.M., the treated leaves or papers and respective controls were introduced into the cages, and the butterflies were allowed to oviposit until the beginning of the afternoon. At 2:00 P.M., the leaves were removed, and the number of eggs was counted. The experiments were carried out in several cages per day and 3–4 d per treatment with new pairs of butterflies each day, adding up to a total of 24–36 independent replicates.

Surface Application of JA The effect of JA-induced changes in Brussels sprouts plants on butterfly behavior was tested in oviposition experiments with *P. brassicae* and *P. rapae*. Three concentrations of JA solution, 0.01, 0.1, and 1 mM, corresponding to approximately 1.25, 12.5, and 125 μg JA/g fresh weight (or 0.25, 2.5, and 25 nmol JA/cm^2) respectively, were sprayed on the plants and tested against a control (plants treated with 0.1% Tween 20). The next morning, just before the start of the experiment, the fourth, fifth, and sixth leaves from the base of the plants were cut, and their petioles were placed directly into a vial with tap water and introduced into the cages with butterflies.

Systemic Uptake of JA For *P. rapae*, two application methods were used to assess the effect of JA-induced changes in Brussels sprouts on oviposition preference. For the second application method, the fourth, fifth, and sixth leaves were cut from untreated plants and placed in a 0.1-mM aqueous JA solution 22 hr before the start of the experiment. Total uptake of the solution was on average 6.3±1.5 ml per control leaf and 6.0±1.6 ml for JA-treated leaves (corresponding to approximately 20 μg JA/g fresh weight or 5 nmol JA/cm^2, assuming homogeneous distribution over the leaf tissue after uptake).

Effect of Pure JA on Oviposition Preference In the next experiment, green cardboard paper sprayed with an oviposition stimulant was used to test the effect of pure JA on the oviposition behavior of *P. rapae* on an inert substrate. Sinigrin has been shown to be a suitable oviposition stimulant for *Pieris* butterflies (Van Loon et al., 1992a) and was therefore used to stimulate oviposition on the artificial substrate in this experiment. The paper (8×11.5 cm) was treated with 1 ml of a 5-mM sinigrin solution (Janssen Pharmaceutica, Tilburg, The Netherlands) by spraying it with a Desaga chromatographic sprayer (Heidelberg, Germany). Subsequently, after drying, papers were sprayed with either 1 ml of a 1-mM JA solution or water (control substrates) just before the test (210 μg JA/carton or 11 nmol JA/cm^2).

Bioassays with Purified Glucosinolate (GLS) Fractions GLS were extracted from the leaf surface as described for the chemical analysis. For each treatment, control and 0.1 mM JA, 60 plants were used for the extraction, of which four to five leaves per plant were dipped. Subsequently, the extracts were fractionated following the protocol of Sørensen (Sørensen, 1990). The GLS fractions were stored in the freezer until analysis. The GLS were dissolved in methanol to make two concentrations, one corresponding to the amount of GLS extracted from the material of two plants in 0.8 ml and one concentration corresponding to the amount of GLS from one leaf in 0.8 ml. Hereafter, we will express these concentrations in gram leaf equivalents (gle). One gle corresponds to the amount of GLS extracted from 1 g fresh and intact leaf. With an average weight of 6 g per leaf, the highest concentration corresponds to 48 gle and the lower concentration to 6 gle. With a sprayer, a volume of 0.8 ml of one of the solutions was sprayed on green paper following the same method as described above for the test of pure JA. *P. rapae* butterflies were offered a two-choice situation, with one paper sprayed with GLS extracted from control plants and one paper with GLS from JA-treated plants.

Performance of P. rapae Caterpillars The development of first instar caterpillars to pupae was observed on control and JA-treated plants. Control plants were sprayed with a 0.1% Tween 20 solution and JA-treated plants with a solution of 0.5 mM JA with 0.1% Tween 20. Thirty newly hatched *P. rapae* caterpillars were evenly distributed over two plants per treatment, 24 hr after treatment, and were placed in cages (67×50×75 cm) in a greenhouse compartment at 22–24°C and 50–70% RH. The plants were replaced with new plants twice a week, so that the maximum time between induction and larval feeding never exceeded 5 d. The number of days until pupation and pupal weight were recorded.

Statistical Analyses Each individual butterfly female was subjected to a two-choice situation, in which most individuals oviposited on both control and JA-treated leaves. As the egg load differed between individuals, the number of eggs on each treatment per individual was treated as a paired sample. The oviposition data for *P. rapae* were normally distributed; therefore, they were analyzed with a paired *t*-test. The oviposition data for *P. brassicae* were not normally distributed, and therefore, analyzed with the nonparametric equivalent of the paired *t*-test, the Wilcoxon matched-pair signed-ranks test. The data on the developmental time of the caterpillars in the performance test were not normally distributed and were analyzed with a Mann–Whitney *U* test for differences between the treatments. Pupal weight was normally distributed and analyzed with an analysis of variance (ANOVA). Changes in GLS content were analyzed with a Mann–Whitney *U* test. Statistical analyses were performed with SPSS 11.0.

Table 1 Glucosinolate content in surface extracts of *Brassica oleracea* leaves in pmol/cm^2 for control and JA-treated plants

Compound	Control Treatment Median (range)[a,b]	JA Treatment Median (range)[a,c]	*Z*	*P*
Glucoiberin	10.7 (6.3–21.7)	0 (0–6.0)	−2.713	0.007
Sinigrin	9.3 (0–23.5)	4.2 (0–6.8)	−1.774	0.076
4-Hydroxyglucobrassicin	0.7 (0–6.1)	0 (0–0)	−2.207	0.027
Glucobrassicin	34.9 (18.4–98.2)	88.6 (38.6–132.5)	−1.479	0.139
4-Methoxyglucobrassicin	0.5 (0–2.5)	0 (0–1.3)	−1.370	0.171
Total amount of glucosinolates	64.1 (27.0–155.7)	90.0 (42.9–138.8)	−0.563	0.573

[a] Interquartile range from first to third quartile

[b] $N=10$

[c] $N=11$

Results

Chemical Analysis Five GLS were detected in *B. oleracea* leaf surface samples: glucoiberin, sinigrin, 4-hydroxyglucobrassicin, glucobrassicin, and 4-methoxyglucobrassicin (Table 1). No significant difference was detected between JA-treated and control leaves for the total amount of GLS per cm^2. The amounts of glucobrassicin, the most abundant glucosinolate in these samples, 4-methoxyglucobrassicin, and sinigrin, did not significantly differ between control and JA-treated leaves. The amounts of glucoiberin and 4-hydroxyglucobrassicin collected in the leaf surface extracts were significantly lower for JA-treated leaves compared to control leaves (Table 1). The same results were obtained when calculated for the GLS content expressed as nmol per mg dry weight (not shown).

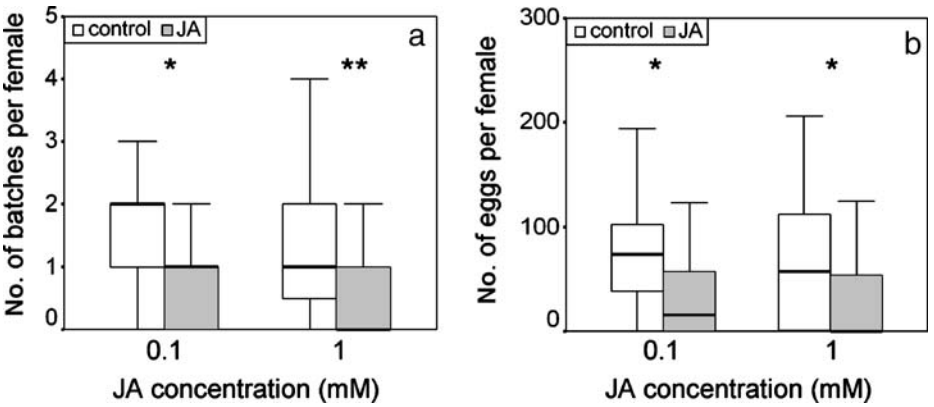


Fig. 1 *Pieris brassicae* oviposition on control and JA-treated plants. Two concentrations of JA were tested against a control. The box represents the interquartile range from first to third quartile, the line across the box indicates the median; *asterisks* indicate statistical differences between the preference for control and JA-treated plants (*$P<0.05$, **$P<0.01$, Wilcoxon matched-pair signed-ranks test). **a** Egg batches per female per leaf. **b** Eggs per female per leaf

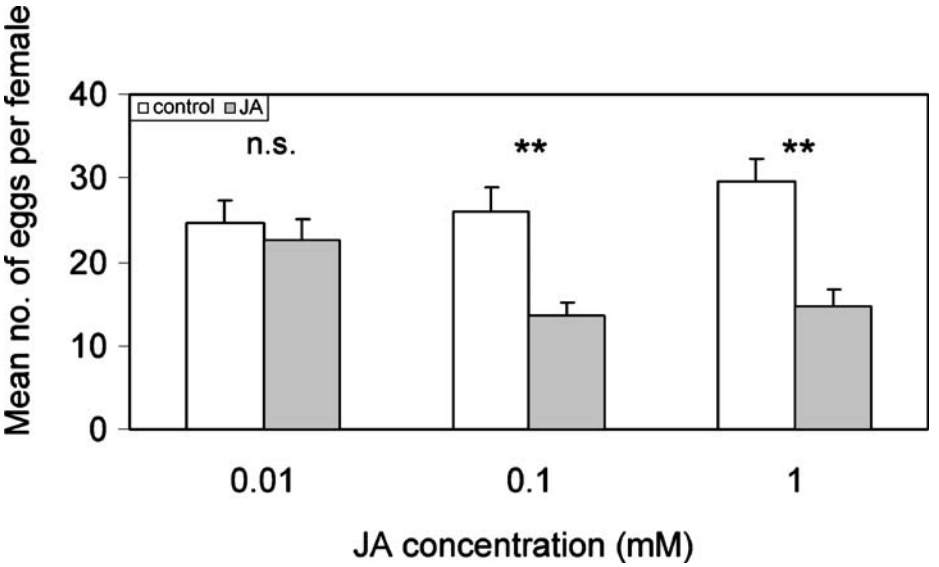


Fig. 2 *Pieris rapae* oviposition preference (measured as the number of eggs per female per leaf) between control and JA-treated *B. oleracea*. Three concentrations of JA were tested against a control in 24 replicated experiments for each concentration. Mean numbers of eggs per female + SEM are given; *asterisks* indicate statistical differences between the preference for control and JA-treated plants (n.s. $P>0.05$, $^{**}P<0.01$, paired *t*-test)

Herbivore Oviposition Preference: Surface Application of JA As *P. brassicae* lays its eggs in batches, both the number of egg batches and the number of eggs per leaf were counted. For the 1 mM JA treatment, the number of batches was significantly lower on JA-treated leaves than on control leaves ($N=36$, $Z=-2.628$, $P=0.009$, Wilcoxon matched-pair signed-ranks test), and the total number of eggs was significantly lower as well ($N=36$, $Z=-2.035$,

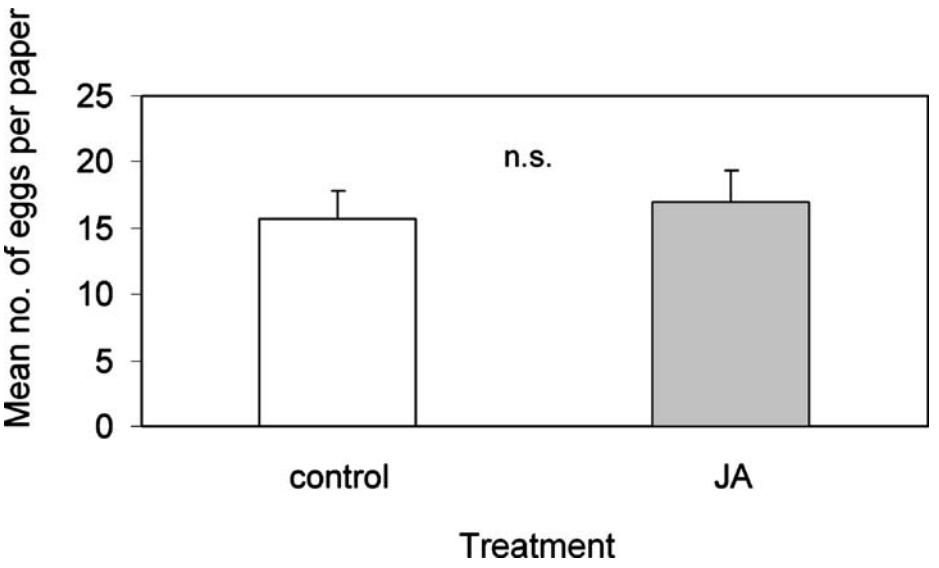


Fig. 3 Oviposition of *P. rapae* on green paper sprayed with sinigrin plus JA and green paper with sinigrin only. Mean number of eggs per female per paper leaf + SEM (n.s. $P>0.05$, paired *t*-test, $N=27$)

Table 2 Glucosinolate (GLS) content in fractions from leaf surface extracts from control and JA-treated plants in pmol/cm^2, *Z* and *P*-values of Mann–Whitney *U* test

Glucosinolate	GLS from control plant	GLS from JA-treated plant
Glucoiberin	4.9	3.9
Sinigrin	3.1	5.1
4-Hydroxyglucobrassicin	Not detected	Not detected
Glucobrassicin	16.0	81.0
4-Methoxyglucobrassicin	Not detected	0.8

P=0.042, Wilcoxon matched-pair signed-ranks test). For the 0.1-mM JA treatment, the result was similar (*N*=27, batches: *Z*=−2.223, *P*=0.026; eggs: *Z*=−2.138, *P*=0.032, Wilcoxon matched-pair signed-ranks test) (Fig. 1). Experiments with 0.01 mM JA application did not show discrimination by the butterflies between the treated and control leaves (results not shown). Also, *P. rapae* butterflies significantly preferred to oviposit on control leaves compared to JA-treated leaves (Fig. 2). The leaves treated with the two highest concentrations of JA tested, 1 and 0.1 mM, were avoided in favor of the control leaves (paired *t*-test, respectively, *t*=3.805, *df*=23, *P*=0.001 and *t*=3.681, *df*=23, *P*=0.001). The lowest concentration of JA tested, i.e., 0.01 mM, did not affect the distribution of eggs over the leaves (*t*=−0.662, *df*=23, *P*=0.52, paired *t*-test).

Systemic Uptake of JA In the experiment with *P. rapae* that employed systemic uptake of JA through the petiole, the same result was obtained: the number of eggs on the JA-treated leaves (10.0±1.57) was lower than on the control (19.12±2.82) leaves (*t*=3.976, *df*=31, *P*<0.001, paired *t*-test).

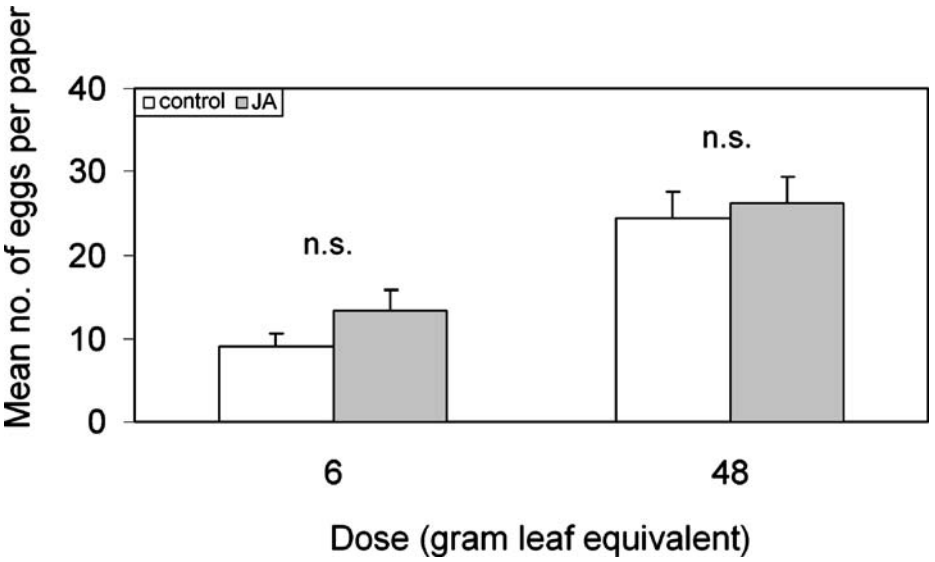


Fig. 4 Oviposition of *P. rapae* on green paper sprayed with purified GLS-fractions from leaf surface extracts of control and JA-treated plants. Mean number of eggs per female per paper leaf + SEM (n.s. *P*>0.05, paired *t*-test, *N*=20)

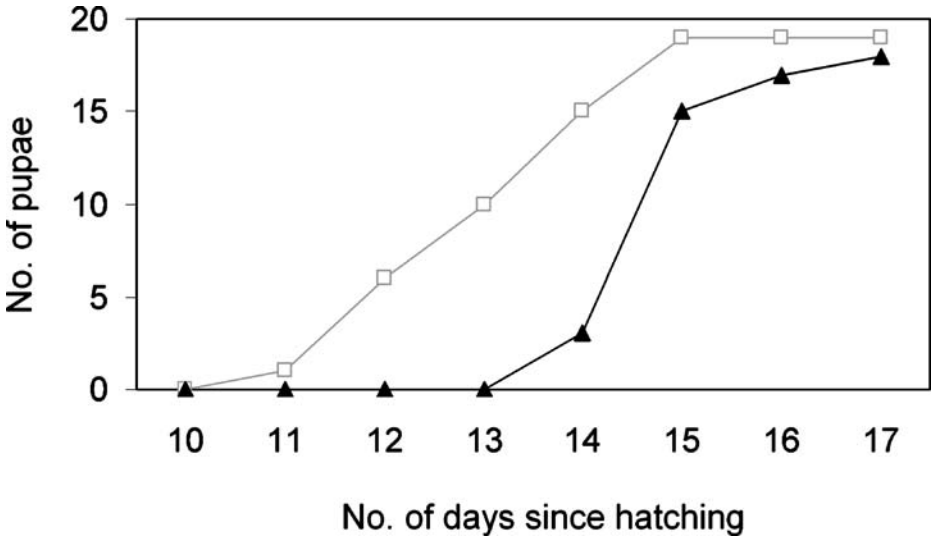


Fig. 5 Development time of *P. rapae* caterpillars from hatching until pupation on control [*open squares* (□)] and JA-treated [closed triangles (▲)] plants. Cumulative number of pupae per treatment

Effect of Pure JA on Oviposition Preference When paper treated with sinigrin and JA was compared to paper with only sinigrin, there was no difference in the number of eggs the butterflies deposited on the two substrates (t=−0.438, df=26, P=0.67, paired t-test) (Fig. 3). These results show that the observed effect of the JA treatment on herbivore oviposition behavior was due to induced changes in leaf tissue rather than to a direct repellent or deterrent effect of JA itself.

Bioassays with Purified Glucosinolate Fractions The butterflies did not discriminate between the two GLS fractions (Table 2), the number of eggs on paper with the GLS from control plants, and the number of eggs on paper with GLS from JA-treated plants was not different for either concentration (concentration 6 gle: Z=−1.514, N=20, P=0.130, Wilcoxon matched-pair signed-ranks test; 48 gle: t=−0.523, df=19, P=0.607, paired t-test) (Fig. 4).

Performance of P. rapae *Caterpillars* About two-thirds of the caterpillars survived until pupation, and a similar number of caterpillars reached the pupal stage on both treatments, 19 on control and 18 on JA-treated plants (Fig. 5). The caterpillars on JA-treated plants pupated on average after 15 d, while the caterpillars on the control plants pupated significantly sooner, on average after 13 d (Mann–Whitney U, Z=−4.071, P<0.001). The average pupal weight on the control plants, 165±3.4 mg, was similar to that on the JA-treated plants, 158±3.3 mg (ANOVA, F=2.665, df=1, P=0.112).

Discussion

Our data show that JA treatment of Brussels sprouts leaves reduces the acceptance of leaves for oviposition by *P. rapae* and *P. brassicae* in a similar way. Treatment of leaves with 0.1 or

1 mM JA reduced the proportion of eggs the butterflies laid on these leaves. A concentration of 0.01 mM JA did not change oviposition preference. The former concentrations are comparable to the concentrations of JA or MeJA that were applied to several plant species in other studies and that reduced development of *Spodoptera exigua*, *Trichoplusia ni*, *Manduca sexta*, thrips, and aphids (Thaler et al., 1996; Avdiushko et al., 1997; Van Dam et al., 2000; Omer et al., 2001), and abundance of *Manduca quinquemaculata*, *S. exigua*, thrips, and flea beetles in the field (Kessler and Baldwin, 2001; Thaler et al., 2001). For cabbage plants, Lu et al. (2004) found inducible resistance in a susceptible *Brassica* species (Chinese cabbage, *Brassica campestris* L.) and induced susceptibility in a resistant *Brassica* species (common cabbage, *B. oleracea*) for *Plutella xylostella* L.

To exclude that JA itself caused the above effect, we tested the phytohormone on an inert substrate and studied two different application methods to the leaf material. We considered this an essential control that is lacking in other studies. The results of these experiments show that it was not JA itself that caused the difference in oviposition preference between control and JA-treated leaves, thus providing proof that processes in the plant induced by the JA treatment changed the acceptability of the leaves. It has also been reported previously that development of cabbage looper or tobacco hornworm larvae is not affected when MeJA is added to an artificial diet, but it is retarded when MeJA was applied to cabbage or tobacco plants (Avdiushko et al., 1997).

In leaf surface extracts of JA-treated and untreated Brussels sprouts, we found five glucosinolates. After JA application, glucobrassicin, the major glucosinolate in the *B. oleracea* cultivar we used, occurred at a level twice as high as in control plants, and glucoiberin and 4-hydroxyglucobrassicin concentrations decreased. The total glucosinolate content did not change significantly. However, most other studies on glucosinolate content in Brassicaceous plants after induction by JA- or MeJA-treatment or insect attack have reported an increase in glucosinolates, although there is substantial variation among different species, or even genotypes, and type of induction (Bodnaryk, 1994; Cipollini and Sipe, 2001; Mikkelsen et al., 2003; Mewis et al., 2005). Moreover, glucosinolates may not be evenly distributed throughout the leaf. We measured glucosinolate content in a surface extract after 24 hr, whereas most studies have measured glucosinolate content in whole leaf extracts and after a longer induction time. Recently, Reifenrath et al. (2005) postulated that the wax layer does not contain glucosinolates, and the polar glucosinolates that are found by using the solvent extraction method are washed from the inner leaf to the outside through the stomata. Nevertheless, we chose a surface extraction method because the butterflies retrieve chemosensory information from the leaf surface, as they do not damage the leaf before ovipositing. Surface extracts are thus likely to give a better reflection of the chemosensory information used than whole leaf extracts.

Both butterfly species distinguish between induced and non-induced leaves, most likely based on chemical differences, as JA-induced leaves do not display herbivore presence or damage. The different levels of two out of five glucosinolates in the surface extracts may provide a chemosensory basis for the oviposition preference observed, although the isolated GLS from the two treatments yielded no differences in acceptance of the paper for oviposition. While the isolated GLS on paper stimulated oviposition behavior, they appear not to be the main cue to discriminate between the JA-induced and non-induced cabbage plants.

We did not quantify other chemicals, stimulants, deterrents, or precursors that might mediate preference behavior, such as isothiocyanates, terpenoids, other glycosides, or amino acids (Huang et al., 1993; Renwick and Chew, 1994; Soldaat et al., 1996; Agrawal and Kurashige, 2003). Both *P. rapae* and *P. brassicae* can perceive a broad range of chemicals (Van Loon et al., 1992b; Hern et al., 1996). Electroantennogram responses to a

range of plant volatiles are similar for both species (Van Loon et al., 1992b), although host plant selection by *Pieris* butterflies appears largely based on contact chemoreception rather than olfaction (Renwick and Chew, 1994). Host plant selection is suggested to depend on a balance of stimulants and deterrents and not just on the detection of presence or absence of particular compounds (Huang et al., 1993; Bruce et al., 2005). Therefore, glucosinolates, in combination with other stimulants or deterrents, may determine the acceptance of a host plant by the butterflies.

The octadecanoid pathway, in which JA is a key molecule, is involved in induction of synomones in response to oviposition and to herbivore damage (Meiners and Hilker, 2000; Dicke and Van Poecke, 2002; Hilker and Meiners, 2006; Mumm and Hilker, 2006). JA treatment of plants results in emission of synomones that attract natural enemies like predatory mites and parasitoids (Dicke et al., 1999; Hilker and Meiners, 2002; Van Poecke and Dicke, 2002; Hilker and Meiners, 2006). This attraction results in a higher natural enemy density around damaged plants, and therefore, it is advantageous for the herbivores to avoid oviposition on induced plants. Moreover, intact plants lack competitors, either intra- or interspecific. Herbivores may use induced plant cues to detect the presence or absence of other herbivores, especially because plant cues are, although less reliable, often easier to detect than cues from the herbivores themselves (Vet and Dicke, 1992).

Furthermore, induced plants may affect herbivores directly by influencing the performance of their offspring. For *P. rapae*, the development time differed between caterpillars feeding on JA-induced and caterpillars feeding on non-induced plants. Development of the caterpillars to pupae took longer on the induced plants, which exposes them to natural enemies for a longer time and gives them a disadvantage in the competition for resources with other herbivores. These results comply with those of Agrawal and Kurashige (2003), who showed that growth of *P. rapae* larvae was reduced on herbivore-induced Brassicaceae.

In summary: (1) JA treatment of *B. oleracea* results in avoidance of host plants by the two *Pieris* butterflies; (2) the related gregarious and solitary butterfly species tested responded in a similar fashion to JA-treated plants; (3) JA treatment reduced the contents of two out of five glucosinolates in leaf surface extracts of Brussels sprouts; and (4) the purified GLS fractions could not explain the observed avoidance behavior. The results indicate that JA-induced infochemicals play an important role in host plant selection behavior of these butterflies; however, the phytochemicals involved still have to be elucidated.

Acknowledgments We thank Niels Agerbirk for advice on GLS isolation; Ciska Raaijmakers for technical assistance with the chemical analysis; Leo Koopman, Frans van Aggelen, André Gidding, and PPW for the supply of insects and plants; Erik Poelman, Tjeerd Snoeren, and Adriana Alvarez for comments and discussion; and the undergraduate students who participated in some of the experiments: Paul Egan, Eloy Erro, Olivia Garcia Gonzales, and Pascal van der Sandt. This work was supported by a VICI grant from the Netherlands Organization for Scientific Research, NWO (865.03.002).

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J Chem Ecol (2007) 33:669–681
DOI 10.1007/s10886-007-9254-9

Systemin Regulates Both Systemic and Volatile Signaling in Tomato Plants

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Received: 29 October 2006 / Revised: 16 December 2006 / Accepted: 17 January 2007 /
Published online: 28 February 2007
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Abstract The prevailing reaction of plants to pest attack is the activation of various defense mechanisms. In tomato, several studies indicate that an 18 amino acid (aa) peptide, called systemin, is a primary signal for the systemic induction of direct resistance against plant-chewing pests, and that the transgenic expression of the *prosystemin* gene (encoding the 200 aa systemin precursor) activates genes involved in the plant response to herbivores. By using a combination of behavioral, chemical, and gene expression analyses, we report that systemin enhances the production of bioactive volatile compounds, increases plant attractivity towards parasitiod wasps, and activates genes involved in volatile production. Our data imply that systemin is involved in the systemic activation of indirect defense in tomato, and we conclude that a single gene controls the systemic activation of coordinated and associated responses against pests.

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Keywords Prosystemin · Volatile · Plant–insect interaction · Gene expression · Indirect defense

Introduction

A large number of the plant defenses against pests are inducible and avoid an unnecessary use of metabolic resources in the absence of recurring and frequent attack (Walling, 2000; Heil and Baldwin, 2002). The activation of defense mechanisms following insect attack leads to the production of several toxins and antifeedant proteins, which reduce the fitness of the invader (direct defense), and volatile organic compounds (VOCs), used by natural enemies of phytophagous insects as semiochemicals to locate their victims (indirect defense) (Stout et al., 2006). Interestingly, some direct defense strategies, particularly those that slow herbivore growth by reducing their digestive efficiency, may not increase the fitness of the plants under attack without a third trophic level because slow-growing insects will eat more plant material while completing their development (Kessler and Baldwin, 2002).

Tomato plants and other Solanaceae respond to chewing insects and mechanical wounding by releasing a highly mobile peptide called systemin (Bergey et al., 1996; Schilmiller and Howe, 2005). In tomato, this 18-amino acid (aa) molecule is synthesized as a 200-aa precursor protein named prosystemin (McGurl and Ryan, 1992). After its proteolytic cleavage, the peptide binds a plasma membrane-bound receptor kinase (Scheer and Ryan, 2002). Subsequently, it is assumed that the signaling pathway proceeds via the activation of a phospholipase activity, with the release of linolenic acid and the formation of jasmonic acid (JA) and its derivative, methyl jasmonate (Bergey et al., 1996). Eventually, the transcriptional activation of a number of genes, yet to be fully identified, leads to an increase of compounds (i.e., proteinase inhibitors and polyphenoloxidase) that are directly toxic to phytophagous insects (Bergey et al., 1996). Currently, mutational evidence implies that long-distance defense signaling is transmitted mainly by JA as a wave travelling via a positive amplification loop that involves systemin release (Schilmiller and Howe, 2005).

While it is widely accepted that systemin activates the wound response in undamaged parts of the plant (Ryan and Pearce, 2003), unexpectedly, its possible role in modulating ecological interactions has not been elucidated. In infested plants, it is necessary that a signal be transmitted from the site of damage to distal unwounded leaves to trigger synthesis and the release of VOCs because volatile compounds are synthesized *de novo* at the site of release (Paré and Tumlinson, 1998). However, it is not clear whether there is a signal specific for volatile production (Degenhardt et al., 2003). It has been proposed that at least some plant elicitors of defense genes are likely to boost effects broader than expected, possibly having the role to activate shared resistance responses (Walling, 2000), because for a plant, challenged by different invaders, it is less expensive to have signal transduction pathways that are involved in the coordinated response to pests (Walling, 2000; Kessler and Baldwin, 2002).

Most of the knowledge about plant indirect defenses and the emission of volatiles attractive to parasitoids has been based on plant treatment with chemical elicitors such as JA (Thaler, 1999; Birkett et al., 2000; Howe, 2004; Cooper and Goggin, 2005). Although considerable progress has been made by using exogenously applied compounds, it has been pointed out that artificial chemical treatments rarely mimic natural responses in quantitative terms (Heil and Baldwin, 2002), and they elicit many physiological and morphological changes that are not related to resistance (Heil, 2002). Recently, the roles of some genes in

plant indirect defense mechanisms have been elucidated by direct genetic manipulation. Several studies have focused on the downregulation of gene expression, which results in the lowering of both volatile production and attractiveness towards predators and parasitoids (Vancanneyt et al., 2001; Kessler et al., 2004; Sanchez-Hernadez et al., 2006). Nonetheless, studies that aim at the identification of mechanisms that enhance direct and indirect plant defense are of greatest interest for plant biotechnology and integrated pest management (Degenhardt et al., 2003).

Our aim was to investigate if the plant hormone systemin can increase indirect defense mechanisms via the transcriptional activation of genes involved in the production of volatile compounds. We report that the overexpression of the systemin precursor influences volatile signaling in tomato, indicating that a single plant hormone leads to the systemic activation of coordinated responses. These results support the model that the elicitation of multiple yet associated defensive pathways is likely to be advantageous and may be necessary if plants need to defend themselves adaptively from diverse insect species.

Methods and Material

Plants and Insects Transgenic tomato overexpressing the *prosystemin* cDNA under the control of the 35S RNA CaMV promoter (BBS) and control plants BB (*Solanum lycopersicum* cv. Better Boy) were as described (McGurl et al., 1994). Plants were grown in sterilized soil and maintained in controlled environmental chambers for 3 weeks from sowing at 26+1°C with a photoperiod of 16:8 hr light/dark. The aphid parasitoid *Aphidius ervi* Haliday was reared on one of its natural hosts, the pea aphid *Acyrthosiphon pisum* (Harris), maintained on potted broad bean (*Vicia faba* L.) cv. Aquadulce as described (Guerrieri et al., 2002). Aphid and parasitoid cultures were kept in separate environmental chambers at 20±1°C, 65±5% relative humidity, 18:6-hr light/dark photoperiod. Insect parasitoids for the flight behavior bioassays were reared as synchronized cohorts as reported (Guerrieri et al., 2002).

Flight Behavior Bioassay Towards Plants Flight behavior of *A. ervi* towards tomato plants was measured as relative attractiveness in a single-choice wind tunnel bioassay. Behavioral observations were carried out as described previously (Guerrieri et al., 2002), modified by using a larger experimental glass box (100×50×50 cm). One hundred and fifty parasitoid females were tested for each target by releasing them individually in the odor plume 35 cm downwind from the target. Each female was only used once. Parasitoids were observed for a maximum time of 10 min, and flight behavior data were recorded and analyzed with the aid of event-recording software (the Observer, Noldus Information Technology, Wageningen, the Netherlands). Behavioral experiments were conducted on several days, and targets were presented in a random order to reduce the effect of temporal variability on the results. In a wind tunnel bioassay, 10 uninfested plants of each BB and BBS were tested. The percentage of response (oriented flights, landings on the target) to each target was calculated. The number of parasitoids responding to each target in any experiment was compared by a G test for independence with William's correction (Sokal and Rohlf, 1995). The resulting values of G were compared with the critical values of x^2 (Rohlf and Sokal, 1995).

Volatile Collection and Identification All glassware, silicon, and Teflon connections were scrupulously clean and heated at 100°C overnight before use. Volatiles from BB and BBS plants were collected by an airtight entrainment system immediately after the wind-tunnel

bioassay. Single plants were placed into bell jars (20 l) sealed with Parafilm and connected to a circulation pump whose flow was adjusted at 200 ml/min. Before reentering the pump, the air passed through an adsorbent trap made of Tenax (Cat. no. 226-336, SKC, Eighty Four, PA, USA) connected to the system by a Teflon-capped glass plug. In order to reduce any stress to the plant in the system, each collection lasted 3 hr. Air entrainment volatiles were separated by an integrated system including thermal desorber (Tekmar TD-800), gas chromatograph (column: RTX-200, 60 m, 0.25 mmID, 0.25 μm, carrier gas: He), and mass spectrometer. The resulting peaks were compared with 36 available standards (Appendix) and compound's database (National Institute of Standards and Technology). The mean quantities of these compound collected from 10 plants of each BB and BBS were analyzed by ANOVA, and the resulting values of P were compared with the critical values of x^2 (Rohlf and Sokal, 1995).

Flight Behavior Bioassay Towards Compounds After identification, commercial standards of the individual compounds that were overproduced by BBS plants were diluted in redistilled hexane and tested in a single choice wind tunnel bioassay. Each compound was tested at a concentration of 1 mg/ml. For each compound, a 50-μl solution was placed singly onto a small piece (3×1 cm) of filter paper (Whatman, no.1). This was inserted into an open-ended glass test tube (6×1 cm) with the end facing the parasitoid and surrounded by a green circular target to reduce any negative effect due to the absence of physical stimuli normally provided by the plant. A fresh aliquot of solution and a new piece of filter paper were used for each female parasitoid. A similar target, which was dosed with 50 μl redistilled hexane, served as control. One hundred and fifty parasitoid females were tested in a single-choice bioassay for each compound, under the same general conditions as those described above for the BB and BBS tests. Compounds were tested on different days in different sequences to eliminate any time variation. The percentage of response (oriented flights, landings on the target) to each compound was calculated and compared by a G test for independence with William's correction (Sokal and Rohlf, 1995). The resulting values of G were compared with the critical values of x^2 (Rohlf and Sokal, 1995).

RNA Isolation and Real-Time RT-PCR Total RNA was prepared from leaves as described (van Blokland et al., 1998) and subsequently treated with RNase-free DNase I (Amersham, Buckinghamshire, UK) to remove residual genomic DNA. First-strand cDNA was synthesized according to previously reported procedures (Corrado et al., 2005). Amplification of the cDNA coding for the *Elongation Factor 1-α* gene, a ubiquitously expressed gene (Shewmaker et al., 1990), served as control for cDNA synthesis and PCR efficiency in the different samples. Moreover, the sequences annealed by the two primers StEF fw and LeEF rv are localized in two contiguous exons for the detection of possible contaminant DNA in the PCR amplifications. Real-time PCRs were performed by using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions (total volume of 25 μl) were prepared with 12.5 μl of the 2× SYBR Green PCR Master Kit (Applied Biosystems), 0.3 pmol of a primer pair, and 0.2 μl of cDNA template. Three independent amplifications were performed from each cDNA sample, and reactions were done in triplicates. The thermal cycling program started with a step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of a 15 sec step at 95°C followed by 1 min at Ta indicated in Table 1. To check the specificity of the amplification products, a dissociation kinetics analysis was performed after each assay. Reaction products were also resolved onto an agarose gel to verify amplicon size. The primer pairs used and the size of the expected amplicons are shown in Table 1. Primers, designed with the aid of the Primer Express 2.0

Table 1 Gene-specific primers used in real-time RT-PCR and relevant parameters

Primers	Gene	Sequence (5′ to 3′)	% GC	Amplicon Length (bp)	Ta	Accession Number
StEF fw	*EF 1-α*	GCTCCAGTGCTTGACTGTCAC	56	420	54	X53043
LeEF rv		CACAACCTAATACACAACTC	40			
BB-BBS fw	*Prosystemin*	GGGAGGGTGCACTAGAAATAA	47	110	51	M84800
BB-BBS rv		TTGCATTTTGGGAGGATCACG	47			M84801
PcSISfw RT	*Prosystemin*	TCTGAATTTGTCTCCCGTTAGAA	39	142	58	M84801
PcSISrv RT		AGCCAAAAGAAAGGAAGCAATAC	39			
TomLoxCfw	*TomloxC*	TTGCCTATGGTGCTGAATGGA	48	101	58	U37839
TomLoxCrv		CAAGCCATGTGGTTCATTTGG	48			
TomLoxBfw	*TomloxB*	CAATCAGTTTGTTGTGCTTGT	52	101	58	U63118
TomLoxBrv		AGACAATACAAGACAATACTC	52			
HPL fw	*HPL*	CCAATCGCGGATCGATTAGAC	52	101	58	AF230372
HPL rv		GGCACGTTCGTTCTGAAAACC	52			
EF fw	*EF 1-α*	CAACCCTGACAAAATCCCCTTT	45	95	58	X14449
EF rv		TTGGTCCCTTGTACCAGTCGAG	50			
TomLoxDfw	*TomloxD*	TTCATGGCCGTGGTTGACA	52	103	58	U37840
TomLoxDrv		AACAATCTCTGCATCTCCGG	52			
GCS fw	*GCS*	TTGGTGAAGCCTTAACTCAGCC	50	101	58	035631
GCS rv		GCAAATGGTGGTGTGCATCAT	48			

% GC=percentage of G and C bases in the primer, *Ta*=experimental annealing temperature.

software (Applied Biosystem) were chosen to amplify a fragment between 50 and 150 bp. Relative quantitation of gene expression was carried out using the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001), where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. We used the housekeeping *Elongation Factor 1-α* gene (Shewmaker et al., 1990) as an endogenous reference gene for the normalization of the expression levels of the target genes. The statistical significance of the results was evaluated as already reported (Alfano et al., 2005).

Results

Effect of Prosystemin Overexpression on the Behavior of an Insect Parasitoid and VOC Production In a wind tunnel test, plants overexpressing the systemin precursor (BBS) proved to be significantly more attractive to naïve females of the aphid parasitoid *A. ervi* than untransformed control plants (BB) (Fig. 1). Nearly three times as many parasitoids oriented towards transformed plants and landed on them compared to controls. Remarkably, as parasitoids were never in contact with tomato plants before the assay, this response is innate. The GC-MS analysis of the volatiles collected from BBS and BB plants immediately after the wind-tunnel test revealed that systemin is involved in the production and/or release of biologically active VOC (Fig. 2). We recorded a significant increase in the total production of these compounds in BBS plants (92,974.1, vs. 35,633.6 ng collected from BB; P=0.03, df=1), and among them, eight were identified (the complete list of purified compounds used for identification is reported in the Appendix). Significant differences were not recorded in the production/release of (*Z*)-3-hexen-ol, 6-methyl-5-hepten-2-one, phellandrene, and *trans*-caryophyllene. In contrast, we collected significantly larger quantities of four monoterpenes from BBS plants, β-ocimene, α-pinene, β-myrcene/3-carene, and limonene (Fig. 3). To prove that these identified compounds overproduced by

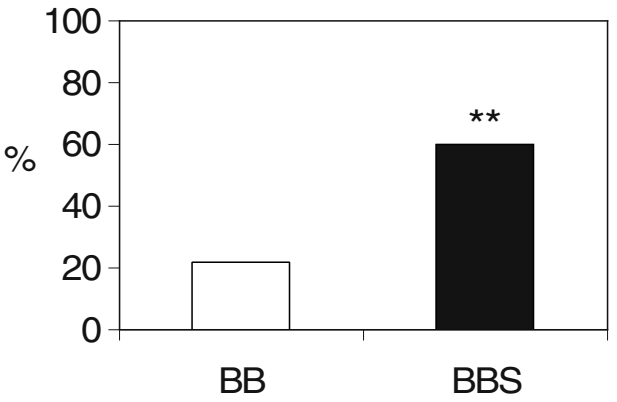


Fig. 1 Flight response (% of female showing oriented flights) of the aphid parasitoid *A. ervi* towards tomato plants in a wind tunnel bioassay (N=150, P<0.001)

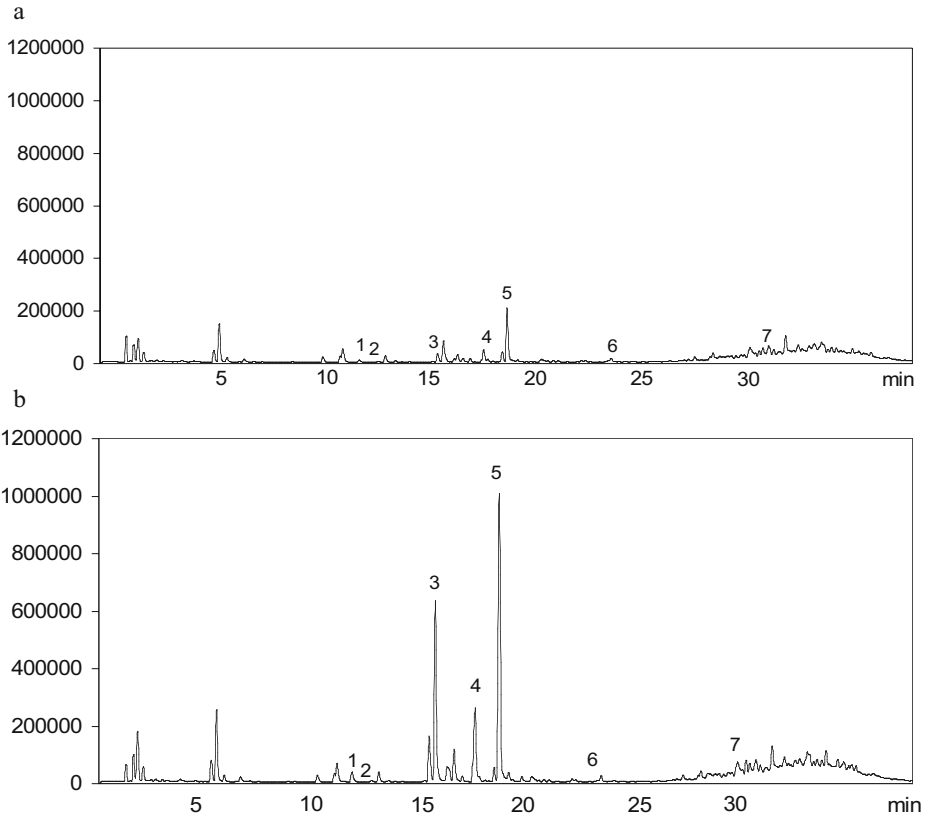


Fig. 2 Chromatograms of volatile emissions collected by air-entrainment of head space from BB (**a**) and BBS (**b**) plants for 3 hr. *Numbers* indicate the identified peaks. *1*, α-pinene; *2*, (*Z*)-3-hexen-ol; *3*, β-myrcene/3-carene; *4*, limonene; *5*, β-ocimene; *6*, 6-methyl-5-hepten-2-one; *7*, trans-β-caryophyllene

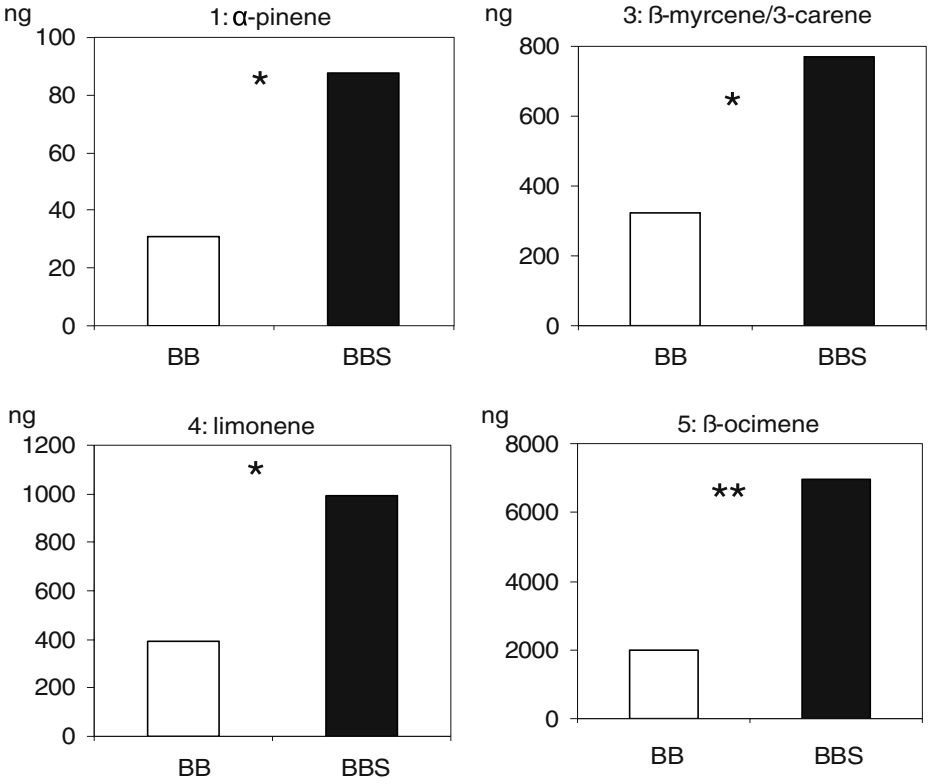


Fig. 3 Quantitative differences of the emission of identified compounds released by BB and BBS plants. Each *panel* shows the mean quantity collected from 10 plants of each BB and BBS expressed in nanograms (*single asterisks*, $P<0.05$; *double asterisks*, $P<0.01$)

BBS plants affect the foraging behavior of the parasitoid *A. ervi*, we assayed α-pinene, β-myrcene, 3-carene, and limonene in a wind tunnel test. Each of these compounds elicited a significant higher proportion of oriented flights by this parasitoid in respect to bidistilled hexane, with α-pinene being the most attractive (Fig. 4).

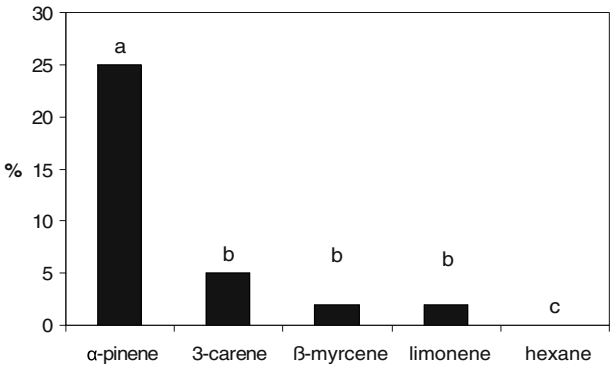


Fig. 4 Flight response of *A. ervi* towards pure volatile compounds in a wind tunnel bioassay expressed as percentage of females showing oriented flights towards the target ($N=150$, *different letters* indicate a statistical difference: *a–b*, $P<0.001$; *b–c*, $P<0.01$)

Expression Analysis Of Genes Involved In Indirect Defense And Volatile Production It has been shown that systemin overexpression increases the transcription of *Lox* genes (Bergey et al., 1996); yet, as there are at least four lipoxygenase in tomato (*TomloxA*, *B*, *C*, and *D*), their role has not been completely clarified in respect to defense mechanisms. We analyzed the expression level of *TomloxC* and *TomloxD*, as their gene products are targeted to chloroplasts, the proposed major site for fatty acid hydroperoxide metabolism. Real-time PCR experiments with gene-specific primers indicated that prosystemin overexpression associates with an increased level of expression of both *TomloxC* and *TomloxD* genes, implying that they have direct roles in plant defense and volatile production in leaves (Fig. 5). On the other hand, the transcription level of the *TomloxB*, which is not involved in the production of the known tomato flavor volatiles (Griffiths et al., 1999), was not affected (Fig. 5). Our results indicate that, in tomato, individual LOX isoforms are differentially regulated and should have distinct functions in systemin-mediated onset of direct and indirect defense mechanisms. The metabolism of linoleic and linolenic acids (the principal LOX substrates) is determined not only by the substrate and product specificities of the lipoxygenases but also by fatty acid hydroperoxide lyases (HPLs) (Matsui et al., 2001), important for the production of a vast class of VOC (Paré and Tumlinson, 1997). The analysis of the level of expression of the *HPL* indicated a moderate relative increase in the amount of transcripts in the BBS plants. Finally, as the analysis of volatile blend in transgenic plants indicated the presence of terpenoids, we also analyzed the expression of *germacrene C synthase* (*GCS*). This gene encodes one of the main enzymes responsible for the sesquiterpene synthase activity in tomato leaf (Colby et al., 1998). Our results indicated that prosystemin overexpression associates with increased levels of transcription of *GCS C*, a key gene involved in the production of terpenoid volatile compounds in tomato.

Only recently has the systemin peptide been proposed as a promoter for long-distance defense responses by supporting the jasmonate production in vascular tissues. Hence, if an important role of the *prosystemin* gene is to generate and amplify a cell-to-cell signal to increase the production of phloem-mobile JAs, *prosystemin* transcription should be subject

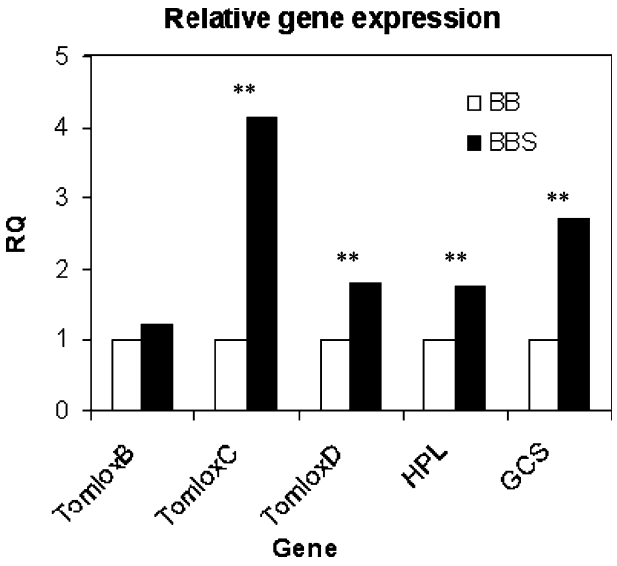


Fig. 5 Relative gene expression by real-time PCR of genes involved in indirect defense against insects. For each gene, the graph displays the relative quantity of the target in BB (*white columns*) and in BBS (*black columns*). Quantities (*RQ*) are shown relative to the calibrator BB genotype. On the *x-axis* is reported the lists of the target genes analyzed. On the *y-axis* is reported the results of the relative quantification calculations. Relative quantities are graphed on a linear scale. *Asterisks* indicate that the $2^{-\Delta Ct}$ values were significantly different ($P<0.01$; Student's *t*-test)

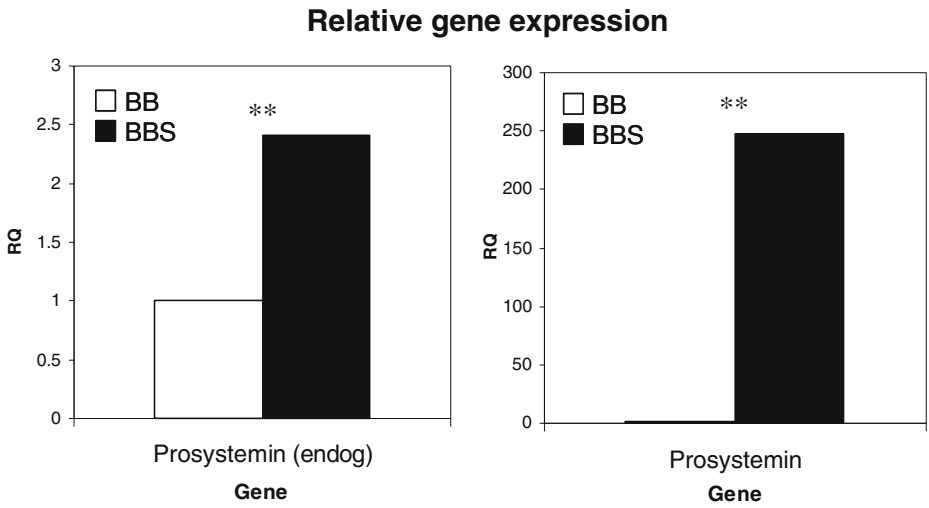


Fig. 6 Relative gene expression of *prosystemin* transcripts by real-time PCR. The graph displays the relative quantity of the target transcripts in BB (*white columns*) and in BBS (*black columns*). *Left panel*: primers were designed to amplify only the transcripts of the endogenous *prosystemin* gene. *Right panel*: primers were designed to amplify both the endogenous and the transgenic *prosystemin* transcripts. Quantity (*RQ*) is shown relative to the calibrator BB genotype. On the *y-axis* is reported the result of the relative quantification calculations on a linear scale. *Asterisks* indicate that the $2^{-\Delta Ct}$ values were significantly different ($P<0.01$; Student's *t*-test)

to a positive enhancement to generate a transmissible signal. We investigated whether the transgenic expression of *prosystemin* cDNA has some transcriptional influence on the endogenous gene. For this purpose, we used two pairs of primers. The pair BB–BBS fw and BB–BBS rv anneals in a region shared by the transgene and the endogenous genes, and hence, they are able to amplify both transcripts. A relative quantification study based on real-time PCR experiments indicated that the amount of *prosystemin* transcripts in transgenic plants is much higher than in unwounded control plants, as expected. The primer PcSISrv RT anneals in a 3′ untranslated region that is absent in the transgenic *prosystemin* cDNA. This primer, in combination with the PcSISfw RT, can amplify only transcripts of the endogenous gene. A relative quantification study indicated that the expression of the endogenous *prosystemin* gene more than doubled in the transgenic BBS (Fig. 6). These experiments indicated that, despite the high level of defensive proteins in transgenic plants constitutively accumulating the systemin precursor, the *prosystemin* overexpression associates with an increase in the expression of the endogenous gene.

Discussion

The identification of genes that can be used to increase plant indirect defenses is of interest to understand plant biology and ecological relations and holds great promise for modern agricultural practices (Degenhardt et al., 2003). While there are different chemicals that, when exogenously applied, can influence tritrophic interactions, only a relatively reduced number of mutants with an altered production of some type of VOC have been identified (Kessler and Baldwin, 2002; Schilmiller and Howe, 2005). Many have obtained down-regulating gene expression by antisense technology (Vancanneyt et al., 2001; Kessler et al.,

2004; Sanchez-Hernadez et al., 2006). A straightforward approach towards the functional characterization of plant genes that can enhance indirect defenses is probably the analysis of genes that are naturally involved in the generation of response against pests. Because both direct and indirect defense should co-operate in nature to increase the fitness of the plants under attack, we investigated the role of *prosystemin* in tritrophic interactions. Different lines of evidence indicated that *prosystemin* overexpression associates with increased indirect defenses in tomato. Although we cannot exclude that additional factors, such as oral secretions, can influence, in a more complex way, plant gene expression and VOC production in undamaged leaves (Alborn et al., 1997; Maffei et al., 2004), our work indicates that the primary signal molecule systemin controls overlapping transduction pathways for different resistance mechanisms. The presence of a plant signal that coordinates the mounting of both direct and indirect defense implies that the attraction of a third trophic level is a relevant function of nonspecifically induced plant volatiles. This is in agreement with the finding that long-lasting mechanical wounding on defined leaf areas (in absence of herbivores) is sufficient for the emission of an herbivore-like VOC blend (Mithofer et al., 2005). The broad-role of systemin here described means that even pests completely devoid of salivary secretion or producing chemicals that are not recognized by the plant response systems would eventually trigger coordinated defense mechanisms.

While it has been demonstrated previously that jasmonate-inducible plant defenses cause increased parasitism of herbivores (Thaler, 1999), our results indicate that the effects of nonspecifically induced volatiles are more complex than expected. The response of *A. ervi* to the VOC induced by *prosystemin* overexpression must be considered innate, as the parasitoids used in a wind tunnel bioassay were obtained from a different host and, thus, have never been in contact with tomato plants. It has been recently reported that tomato plants respond to biotic stresses altering the profile of VOC only in a quantitative way (Ament et al., 2004; Kant et al., 2004; Sanchez-Hernadez et al., 2006), and the effect of the systemin is consistent with those observations (see Fig. 2). All of the compounds that are overproduced/released by tomato plants that overexpress *prosystemin* seem to play a role in the attractiveness towards *A. ervi*. Apart from β-ocimene, which has been tested previously at the same concentration and that elicited a very high response (Birkett et al., 2000), among the four remaining compounds overproduced/released by BBS plants, α-pinene was the most active, followed by limonene, 3-carene, and β-myrcene (Fig. 4). The release of these compounds appears to be associated to plant responses to unspecific stresses, including aphid infestation (Sasso et al., unpublished), and this could explain the positive response recorded in an aphid parasitoid. Moreover, the lower attractiveness (expressed as percentage of oriented flights) that single compounds elicited in respect to the bioassay with plants, was expected as a consequence of the partition of the volatile bouquet. Furthermore, it is also possible that other unidentified peaks can also be involved in multitrophic interactions. As a consequence, for engineering volatile emission to make crop plants more attractive towards pests enemies, genes that code primary defensive signals may be preferred to those that control a single or a limited set of volatile compounds (Degenhardt et al., 2003).

While our data are consistent with an important role for the octadecanoid-related pathways in enhancing multiple and associated response to pests (Walling, 2000; Schilmiller and Howe, 2005), they contrast with a recent publication indicating that, despite that the *spr2* tomato mutant (impaired in the JA biosynthesis) has a reduced level of indirect defense, *prosystemin* overexpression associates with a marginal difference in VOC production (Sanchez-Hernadez et al., 2006). The authors proposed that this discrepancy may be due to the rather low concentration of JA present in the prosystemin overexpressing plants, sufficient to elicit only direct defense genes. This is supported by the finding that

two genes that encode enzymes required for the synthesis of JA (*AOC* and *OPR3*) presented similar very low expression levels (almost undetectable for *OPR3*) in the unwounded 35S::prosys and control plants (Sanchez-Hernadez et al., 2006). However, it is puzzling that such level of expression is also present in the JA-deficient mutant *spr2* (Sanchez-Hernadez et al., 2006), probably suggesting that the activity of these genes is not sensitive to the variation of JA concentration *in planta*. Additionally, in our system, volatiles were collected from the whole plant, including the pot, as bioactive VOC can be produced even from the belowground part of the plant (Rasmann et al., 2005) and for a 6-fold-longer period of time (3 hr). Finally, in agreement with our data, almost all the compounds identified by Sanchez-Hernadez et al. (2006) are present in the list of volatiles collected in this work, with one, namely, β-myrcene, being significantly higher in the plant overexpressing systemin.

Having verified the *prosystemin* overexpression in the transgenic plants and proved that also the endogenous *prosystemin* gene is more active than in control plants, we analyzed the level of expression of genes that have been experimentally linked to volatile production in tomato by using the more sensitive real-time PCR assay. Consistent with the chemical analysis of the volatile blend, some genes responsible for the production of different VOC (i.e., members of the *Lox* family, *HPL*, *GCS*) are overexpressed in the transgenic plants. Our data also indicate the presence of at least a dual regulation of the LOX isoforms in tomato, as not all *Tomlox* genes are involved in systemin-mediated response to biotic stress. For instance, we showed that *TomloxC*, a gene that has been considered important during fruit development for its contribution to aroma and flavor generation (Chen et al., 2004) and whose expression in leaves is undetectable by Northern assay (Heitz et al., 1997), may play a role in the production of volatile defense compounds in leaves. It is not surprising that the same genes perform both roles. The generation of a stronger fruit aroma and the production of VOC involved in indirect defense are two functions that, while aiding seed dispersal and attracting parasitoids, respectively, should increase plant fitness by these diverse biotic interactions.

In summary, our data indicate that systemin is an important element of the whole defensive response in tomato. Considering that some of the identified VOC could participate in plant-to-plant communication (Holopainen, 2004), it would be interesting in the future to address the role of systemin in more complex interactions.

Acknowledgments We thank Clarence Ryan (Institute of Biological Chemistry, Washington State University) for providing the seeds and useful suggestions during manuscript preparation and Adele Cataldo for technical support. This work was supported by Ministero dell'Università e Ricerca Scientifica (PRIN 2004, MIUR Progetto n. 32 Regioni Obiettivo 1).

Appendix

Standards Used for the Identification of Volatiles Collected by Air-entrainment of Head Space from BB and BBS Plants in Alphabetical Order

(+) longifolene, (*Z*)-3-hexen-1-ol, 3-carene, 6-methyl-5-hepten-2-one, anisole-p-allyl, camphor, chlorobenzene, *cis*-nerolidol, decane, dodecene, eucalyptol, eugenol, hexanal, humulene (=α-caryophyllene), linalool, methyl salicilate, menthol, ocimene, p-cymene, p-dichlorobenzene (IS), phellandrene, R(+) limonene, S(−) limonene, skatol, terpinolene, *trans*-caryophyllene, *trans*-nerolidol, *trans*-β-farnesene, α-copaene, α-cubebene, α-gurjunene, α-pinene, α-terpinene, α-terpineol, β-myrcene, γ-terpinene

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J Chem Ecol (2007) 33:683–694
DOI 10.1007/s10886-007-9255-8

Ozone Degrades Common Herbivore-Induced Plant Volatiles: Does This Affect Herbivore Prey Location by Predators and Parasitoids?

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Received: 30 October 2006 / Revised: 18 January 2007 / Accepted: 29 January 2007 /
Published online: 28 February 2007
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Abstract Inducible terpenes and lipoxygenase pathway products, e.g., green-leaf volatiles (GLVs), are emitted by plants in response to herbivory. They are used by carnivorous arthropods to locate prey. These compounds are highly reactive with atmospheric pollutants. We hypothesized that elevated ozone (O_3) may affect chemical communication between plants and natural enemies of herbivores by degrading signal compounds. In this study, we have used two tritrophic systems (*Brassica oleracea–Plutella xylostella–Cotesia plutellae* and *Phaseolus lunatus–Tetranychus urticae–Phytoseiulus persimilis*) to show that exposure of plants to moderately enhanced atmospheric O_3 levels (60 and 120 nl l^{-1}) results in complete degradation of most herbivore-induced terpenes and GLVs, which is congruent with our hypothesis. However, orientation behavior of natural enemies was not disrupted by O_3 exposure in either tritrophic system. Other herbivore-induced volatiles, such as benzyl cyanide, a nitrile in cabbage, and methyl salicylate in lima bean, were not significantly reduced in reactions with O_3. We suggest that more atmospherically stable herbivore-induced volatile compounds can provide important long-distance plant-carnivore signals and may be used by natural enemies of herbivores to orientate in O_3-polluted environments.

Keywords Herbivore-induced terpenes · Green-leaf volatiles · *Cotesia plutellae* · *Phytoseiulus persimilis* · Tritrophic interactions · Ozone

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Introduction

Isoprenoids comprise the predominant group of plant-emitted volatile organic compounds (VOCs) followed by alcohols and carbonyls (Kesselmeier and Staudt, 1999; Owen and Peñuelas, 2005). They have various ecophysiological functions in plants (Peñuelas and Llusià, 2004). They mediate plant–plant (Baldwin et al., 2006; Ton et al., 2007) and several plant–insect (see Owen and Peñuelas, 2005 for a review) interactions. Within the isoprenoids are terpenes, some of which are attractive to phytophagous insects (Ibrahim et al., 2005; Tooker et al., 2005) and evoke electrophysiological responses, including in specialist insects (see Bruce et al., 2005 for a review). They are also attractive to carnivorous arthropods. Many studies have addressed the role of *de novo*-synthesized terpenes (mono-, sesqui-, and homoterpenes) (Paré and Tumlinson, 1999) in the recruitment of herbivore natural enemies, e.g., predators and parasitoids (see Dicke, 1999 for a review; Ibrahim et al., 2005; Kappers et al., 2005; Schnee et al., 2006).

In addition to volatile terpenes, herbivore feeding induces the emission of many lipoxygenase (LOX)-pathway-derived alcohols, esters, and aldehydes from plants (Holopainen, 2004). The emission of these LOX products, commonly referred to as green-leaf volatiles (GLVs), is the result of mechanical damage caused by herbivore feeding. They seem not to be enhanced by elicitors, and, therefore, their release has been described as instantaneous "bleeding" from damage sites. However, two related herbivores can lead to the emission of distinctive ratios of GLVs in the same plant species (Turlings and Wäckers, 2004).

Terpenes and some GLVs can readily react with ozone (O_3) and lead to the formation of many products in the gas phase and the particulate phase (Yu et al., 1999; Calogirou et al., 1999; Atkinson and Arey, 2003; Holzinger et al., 2005). The current concentrations of this tropospheric oxidant show regional and seasonal variations (Calogirou et al., 1996), and they are expected to increase in the coming years (IPCC, 2001). O_3 can affect phytophagous insect performance (Coleman and Jones, 1988; Holopainen et al., 1997; Kopper and Lindroth, 2003) and behavior (Jøndrup et al., 2002; Agrell et al., 2005) due to changes in the plant physiology and chemistry. Few studies have addressed O_3 effects on olfactory cues. O_3 exposure can elicit the emission of herbivore-inducible homoterpenes (Vuorinen et al., 2004a), but terpenes may react with O_3 leading to the destruction of olfactory cues, disrupting insect chemical communication (Gate et al., 1995). This may affect the choices that insects make, thus influencing their reproductive success (Dicke, 2000).

We coupled VOC collections with behavioral tests in Y-tube olfactometers to answer two questions: (1) Can O_3 affect the VOCs in the headspace of intact and herbivore-damaged plants? and (2) Can the oxidation of plant-emitted VOCs disrupt chemical communication between plants and higher trophic levels? We studied two model tritrophic systems. The first was comprised of cabbage plants (*Brassica oleracea* ssp. *capitata*), the oligophagous herbivorous larvae of *Plutella xylostella*, and the solitary hymenopterous endoparasitoid *Cotesia plutellae*. The second was comprised of lima bean (*Phaseolus lunatus*), the polyphagous herbivorous mite *Tetranychus urticae*, and the predatory mite *Phytoseiulus persimilis*. We found that high reactivity and short atmospheric lifetime of terpenes and LOX products in near-ambient (60 nl l^{-1}) and enhanced (120 nl l^{-1}) atmospheric O_3 concentrations resulted in complete degradation of many of these compounds, but the predators and parasitoids still detected herbivore-damaged plants. These results suggest that natural enemies of herbivores might use O_3-stable inducible plant volatiles or degradation products resulting from the oxidation of terpenes that are more stable than their precursors as signals for orientation.

Methods and Materials

Living Material We used standard procedures (Vuorinen et al., 2004a, b) to cultivate cabbage (*B. oleracea* ssp. *capitata* L. cv. Lennox) and lima-bean (*P. lunatus* L.) plants and to rear the herbivores *P. xylostella* L. (Lepidoptera: Yponomeutidae) and *T. urticae* Koch (Acari: Tetranychidae) and the parasitoid *C. plutellae* Kurdjumov (Hymenoptera: Braconidae). The predatory mite *Phytoseiulus persimilis* Athias–Henriot (Acari: Phytoseidae) reared on *T. urticae* feeding on lima bean was supplied by Biotus OY (Forssa, Finland).

Setup for Oxidation of VOCs A main charcoal-filtered airflow of 1.6 l min^{-1} was introduced into an O_3 generator (Model 1008-RS, Dasibi Environmental Corp., CA, USA). The resulting O_3-enriched airflow was divided equally (0.8 l min^{-1}) and channeled into two 22.3-l glass desiccators (Schott Duran, Mainz, Germany) that contained the odor sources. Each odor source consisted of four plants, intact or herbivore-damaged, potted in semirigid polypropene pots, with Teflon covering the soil surface to avoid excess humidity inside the dessicators. The insect feeding damage was accomplished by transferring 10 third-instar *P. xylostella* larvae or 100 *T. urticae* individuals onto two leaves of each cabbage or lima-bean plant, respectively, for 48 hr. The outgoing airflow from each dessicator was branched to give two 0.4 l min^{-1} airflows regulated by airflow meters (NG-LG type, Platon Flow Control, Basingstoke, UK). One branch from each dessicator was diluted with pressurized air, and each was connected to an O_3 analyzer (Model O342M, Environment S.A., Poissy, France) allowing the constant measurement of O_3 in the headspace of each set of plants. Before the experiments, each analyzer was calibrated with the O_3 in the respective dessicator to set target O_3 enrichment levels (0, 60, and 120 nl l^{-1}). The remaining airflow branching from each dessicator was used either to collect volatiles in the headspace of intact and damaged plants or to conduct behavioral tests with the natural enemies (Fig. 1). The whole system was connected by Teflon tubing. Two lamps (PAR, approximately 350 μmol m^{-2} s^{-1}, Model Shuttle Plus, LIVAL, Sipoo, Finland) were placed over the vessels to maintain the optimal photosynthesis levels in the plants.

VOC Collection and Analyses VOCs from the headspace of both intact and damaged plants were collected into tubes containing 150-mg Tenax TA adsorbent (Mesh 60/80, Supelco, PA, USA) at an airflow of 0.215 l min^{-1} for 60 min. The same set of plants was used consecutively for collections at the various O_3 levels. Collections were repeated four times (N=4). O_3 scrubbers [copper tube 4 mm in diameter with an inner bath of potassium iodide (KI)] were placed before the adsorbent to avoid degradation of samples by O_3.

VOCs were analyzed by gas chromatography-mass spectrometry (GC-MS) (Vuorinen et al., 2004b) with a capillary column with a 0.5- and 0.33-μm film for cabbage and lima-bean samples, respectively. The quantification was achieved by comparison with commercially available reference substances or synthesized (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Ibrahim et al., unpublished). α-Thujene, (*E*)-β-ocimene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were calculated by proposing the responses to be the same as the responses of α-pinene, linalool, and DMNT, respectively. Benzyl cyanide and 2-butanone were estimated on the basis of the internal standard 1-chloro-octane. Emission rates in the absence of O_3 were calculated and expressed in nanogram per gram of DW per hour (leaves dried at 60°C for 48 hr). The quantities of chemical compounds in the headspace of plants were calculated in nanogram per liter.

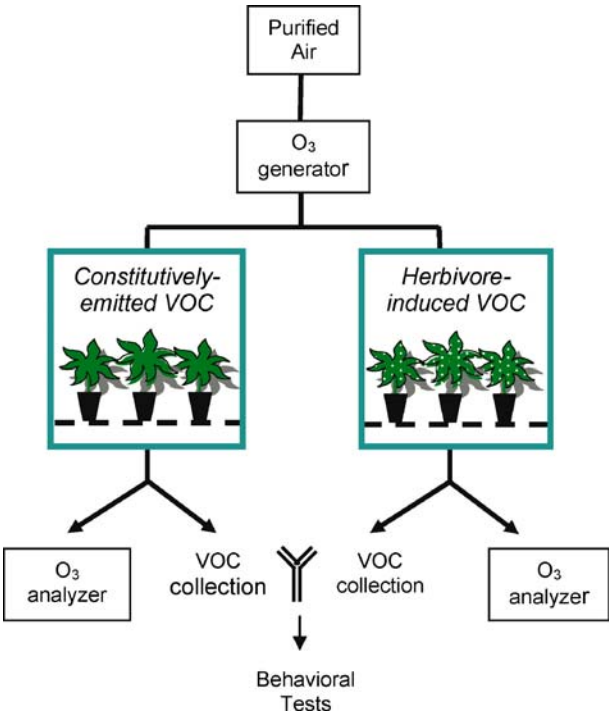


Fig. 1 Experimental setup used for VOC collection and behavioral tests. The O_3-enriched airflow (1.6 l min^{-1}) from the O_3 generator at three concentrations (0, 60, and 120 nl l^{-1}) was channeled equally and directed into each odor source. Outflowing air from the odor source was used for behavioral tests at 0.4 l min^{-1} or VOC collection at 0.2 l min^{-1} and O_3 measurements at 0.4 l min^{-1}

Behavioral Responses of the Natural Enemies Some 3- to 4-d-old *C. plutellae* mated females with a 30-min oviposition experience (Potting et al., 1999) and *P. persimilis* starved for 24 hr individually in Eppendorf tubes at 20°C were used in the bioassays as described in Vuorinen et al. (2004b). Briefly, the wasps or the predatory mites were introduced individually downwind at the end of a Y-tube olfactometer and observed for a maximum of 5 min. "A choice" was recorded when the natural enemy stayed for at least 5 sec further than two-thirds of the way along one of the arms. Otherwise, it was scored as "no choice" after 5 min. Each individual was used once. Tests were conducted under the various O_3 conditions with an airflow of 0.4 l min^{-1}. Two pairs of odor sources were used per day, and 10 observations per O_3 treatment/pair were conducted.

Statistical Analyses Statistical analyses were performed by using the SPSS 11.5 for Windows Statistical Package. The differences between the emission rates from intact and herbivore-damaged plants in the absence of O_3 were analyzed with the Mann–Whitney *U* test. The effect of the O_3 treatments on each individual compound was analyzed by using the nonparametric Friedman rank test for related samples for the headspace of both intact and herbivore-damaged plants. The behavioral responses of the natural enemies were analyzed by using the nonparametric binomial test to test for significant differences in the attraction between the two odor sources (intact vs herbivore-damaged plants) for each O_3 treatment.

Results

VOCs in the Headspace of Cabbage Plants Seven monoterpenes were quantified in the headspace of intact and *P. xylostella*-damaged cabbage plants: α- thujene, α-pinene,

sabinene, β-pinene, β-myrcene, limonene, and 1,8-cineole (Table 1). In the absence of O_3, there were greater emissions of some compounds from herbivore-damaged than from intact plants.

In O_3, the headspace of the intact plants showed reduced levels of α-pinene (P=0.018), sabinene (P=0.039), β-myrcene (P=0.022), and limonene (P=0.018), and increased levels of 1,8-cineole (P=0.018) (Table 2). The headspace of damaged plants showed reductions of α-thujene (P=0.018), α-pinene (P=0.018), β-myrcene (P=0.024), and limonene (P=0.018) and increases of 1,8-cineole (P=0.018). The *P. xylostella*-induced terpenes (Table 1), DMNT (P=0.023), a homoterpene, and (*E,E*)-α-farnesene (P=0.018), a sesquiterpene, decreased significantly in O_3 (Fig. 2a). Some traces of DMNT were detected at both 60 nl l^{-1} (with an exception of one sample) and 120 nl l^{-1} but below the quantification level. Linalool was found only in one sample at 0 nl l^{-1} of O_3, but it was not detected after increasing the O_3. In addition to terpenes, two GLVs, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, and the nitrile, benzyl cyanide, were induced by *P. xylostella* (Table 1). Of these, only quantities of the (*Z*)-3-hexen-1-ol significantly decreased in O_3 (P=0.018), and there was a trend for (*Z*)-3-hexenyl acetate to decrease (Fig. 2a).

VOCs in the Headspace of Lima-Bean Plants The monoterpenes α-pinene and limonene were found in the headspace of intact and *T. urticae*-damaged plants in the absence of O_3 (Table 1).

Table 1 VOC emissions (nanogram per gram of DW per hour) from intact and *P. xylostella* (*PX*)-damaged cabbage and intact and *T. urticae* (*TU*)-damaged lima-bean plants in filtered air

	Cabbage		Lima bean	
	Intact	*PX*-damaged	Intact	*TU*-damaged
Constitutively emitted terpenes				
α-Thujene	1.9±0.6	2.9±0.6	0	0
α-Pinene	1.6±0.0	1.4±0.1	4.3±1.0	3.8±1.0
Sabinene	6.2±1.4	4.9±0.7	0	0
β-Pinene	0.5±0.2	0.3±0.2	0	0
β-Myrcene	3.6±0.4	3.2±0.4	0	0
Limonene	23.9±6.9	33.4±12.2	22.0±4.8	17.7±4.4
1,8-Cineole	2.0±0.4	1.7±0.1	0	0
Inducible terpenes				
(*Z*)-β-Ocimene	0	0	0	6.2±1.2*
Linalool	0	2.1±2.1	0	1.8±1.1
DMNT	0	4.7±0.8*	0	41.0±6.5*
(*E,E*)-α-Farnesene	0	5.2±0.2*	0	0
TMTT	0	0	0	54.8±3.0*
GLVs				
(*Z*)-3-Hexen-1-ol	0	24.8±6.7*	0	3.0±0.5*
(*Z*)-3-Hexenyl acetate	0	30.6±13.3	0	–
Inducible compounds other than terpenes				
Benzyl cyanide	0	4.1±0.5*	0	0
2-Butanone	0	0	0	60.7±6.6*
MeSA	0	0	0	29.7±3.2*

Data are expressed as means±SE (N=4). Significant differences between intact and herbivore-damaged plants are shown with an asterisk (P<0.05).

Table 2 VOC (nanogram per liter) in the headspace of a set of four intact cabbage or lima-bean plants at elevated O_3 concentrations

	0 nl l^{-1} O_3	60 nl l^{-1} O_3	120 nl l^{-1} O_3	*P* value
Cabbage				
α-Thujene	0.50±0.17	0	0	0.050
α-Pinene	0.38±0.04	0.25±0.03	0.19±0.03	0.018
Sabinene	1.57±0.42	1.38±0.34	1.03±0.28	0.039
β-Pinene	0.13±0.05	0.12±0.05	0.14±0.05	0.247
β-Myrcene	0.86±0.10	0.32±0.11	0.12±0.08	0.022
Limonene	6.24±2.43	1.94±0.43	1.49±0.36	0.018
1,8-Cineole	0.05±0.12	0.95±0.26	1.11±0.28	0.018
Lima bean				
α-Pinene	0.28±0.04	0	0	0.018
Limonene	1.64±0.50	0.21±0.16	0.08±0.08	0.024

Data are expressed as means±SE (N=4). *P* values shown by Friedman rank test between O_3 treatments

In O_3, the headspace of intact plants showed reduced levels of α-pinene (P=0.018) and limonene (P=0.024) (Table 2). The headspace of damaged plants also showed reduced levels of α-pinene (P=0.018) and limonene (P=0.018). The *T. urticae*-induced terpenes (Table 1), (*E*)-β-ocimene (P=0.023), a monoterpene, and the homoterpenes DMNT (P=0.039) and TMTT (P=0.022), decreased in O_3 (Fig. 2b). Linalool was induced, but it was only detected in two samples at 0 nl l^{-1}. In addition to the terpenes, the GLV (*Z*)-3-hexen-1-ol, the ketone 2-butanone, and methyl salicylate (MeSA) were also emitted (Table 1). Of these, (*Z*)-3-hexen-1-ol decreased (P=0.023), whereas MeSA increased (P=0.039) in the presence of O_3, and there was a trend for 2-butanone to increase (Fig. 2b). (*Z*)-3-Hexenyl acetate was found at 0 and 60 nl l^{-1}, but the fact that this compound was not separated from octanal on the column and that octanal remained unaffected by O_3 made it difficult to quantify. The traces of this compound disappeared at 120 nl l^{-1}.

Behavioral Responses of the Natural Enemies C. plutellae and *P. persimilis* were able to discriminate between the odor of intact and herbivore-damaged cabbage and lima-bean plants, respectively, in clean air and at both elevated levels of O_3, 60, and 120 nl l^{-1}, showing a highly significant preference for the damaged plants (P<0.001) (Fig. 3).

Discussion

Terpenes with two or more double bonds are readily decomposed by O_3, whereas saturated terpenes, such as 1,8-cineole, remain intact (Calogirou et al., 1996). In our study, O_3 lead to the unequal decomposition of terpenes. In general, they decreased either significantly or showed a trend to decrease in the headspace of both intact and damaged cabbage and lima-bean plants in increasing O_3. Only 1,8-cineole, emitted by cabbage plants, increased with increasing O_3 as previously observed in a reaction chamber (Pinto et al., unpublished results).

Herbivore-inducible terpenes, such as DMNT and TMTT, have been shown to be involved in the attraction of natural enemies by herbivore-damaged plants (Holopainen, 2004). It has been proposed that differences in the amount of DMNT emitted by *P. xylostella*-damaged plants and *P. xylostella*+*Pieris rapae*-damaged plants are responsible

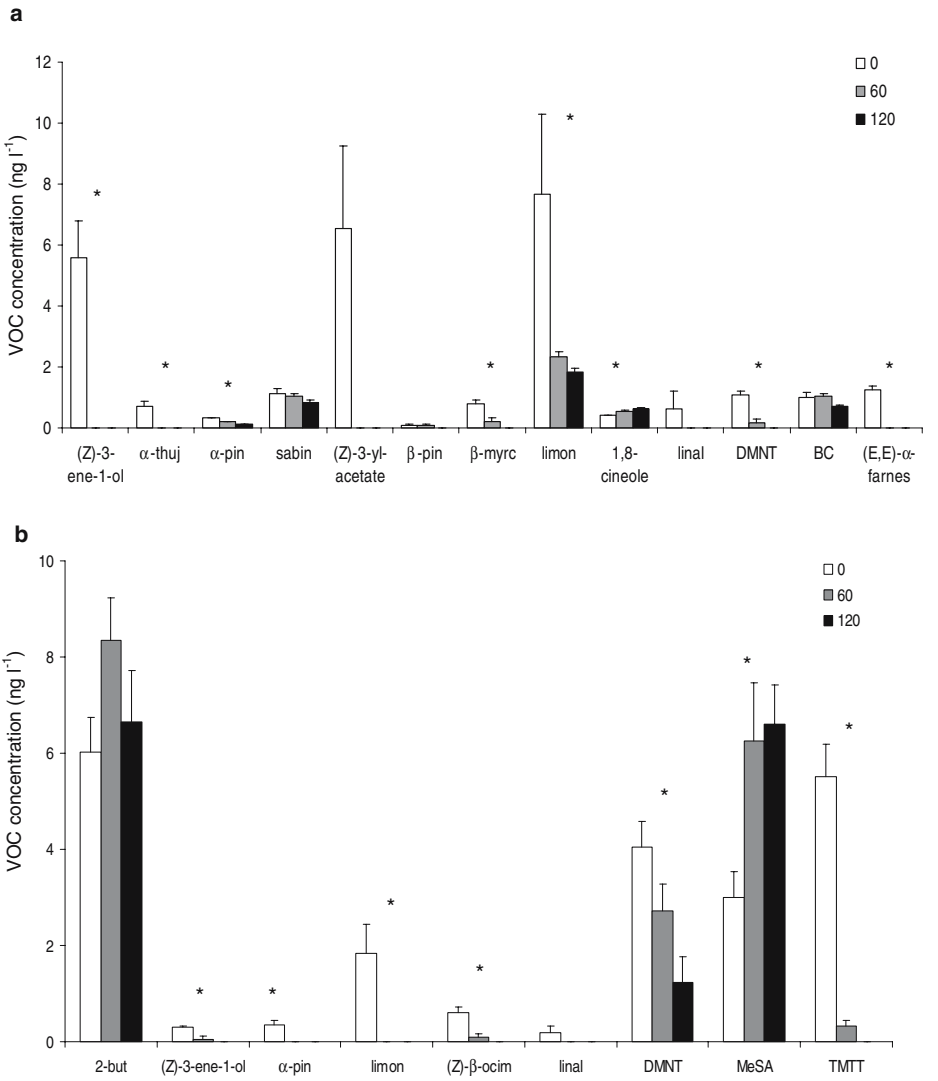


Fig. 2 VOCs (nanogram per liter) in the headspace of **a** a set of four *P. xylostella*-damaged cabbage plants and **b** a set of 4 *T. urticae*-damaged lima-bean plants at different O_3 concentrations. The abbreviations of the compounds refer to those listed in Tables 1 and 2. The amounts of each compound at 0, 60, or 120 nl l^{-1} of O_3 are shown in *white*, *gray*, and *black bars*, respectively, in each series. Data are expressed as means±SE (N=4). *Asterisks* show significance by Friedman rank tests (P<0.05)

for the preferences displayed by *C. plutellae*. *P. xylostella*-damaged plants emit more DMNT than *P. xylostella*+*P. rapae*-damaged plants (Shiojiri et al., 2001). It has been suggested that homoterpenes might also be effectively oxidized and, therefore, contribute to further gas/particulate partitioning of the reaction products (Joutsensaari et al., 2005). In our study, *P. xylostella* and *T. urticae* induced the emission of DMNT. This homoterpene was significantly affected by O_3 after elevating the concentration of the oxidant to 60 nl l^{-1} in the headspace of damaged cabbage plants but not in the headspace of damaged lima bean. However, this compound decreased between 60 and 120 nl l^{-1}. The amount of

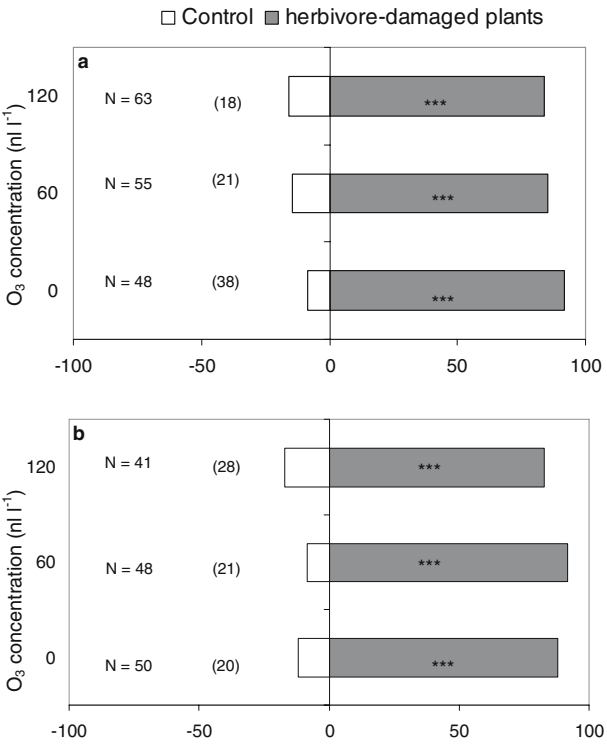


Fig. 3 Behavioral responses of **a** the solitary endoparasitoid *C. plutellae* and **b** the predatory mite *P. persimilis*. The figure shows the percentage of female wasps or predatory mites choosing intact plants (control) or the herbivore-damaged plants in the Y-tube olfactometer at three different O_3-enriched airflows. The significances of the binomial tests ($P<0.001$) are shown with *asterisks* ***. The number of individuals making a choice is shown as "*N*," and the number that did not choose between the two odor sources is in ( ) at the three different O_3 concentrations

DMNT emitted by damaged lima-bean plants was far higher than the amount of DMNT emitted by damaged cabbage plants. TMTT emitted by *T. urticae*-damaged plants was significantly reduced after elevating the O_3. This compound has one more double bond than DMNT, and for this reason, it might be more susceptible to oxidation. *T. urticae*-induced (*E*)-β-ocimene has also been proposed as an important olfactory cue for the predatory mite *P. persimilis*. This compound is emitted by herbivore-damaged but not by O_3-damaged lima-bean plants (Vuorinen et al., 2004a), and it was not detected at 60 nl l^{-1}.

GLVs also elicit behavioral responses in natural enemies, including *C. plutellae* (Reddy et al., 2002), and evoke electrophysiological responses in the antennae of species of the same genus, *Cotesia glomerata* and *Cotesia rubecula*, the parasitoids of the herbivores *Pieris rapae* and *Pieris brassicae*, respectively (Smid et al., 2002). (*Z*)-3-Hexen-1-ol and (*Z*)-3-hexenyl acetate were emitted by both herbivore-damaged plant species. We found that they were severely degraded by O_3. The calculated atmospheric lifetime of these compounds has been estimated to be just a few hours in the presence of O_3 (Atkinson and Arey, 2003). Although we were not able to quantify (*Z*)-3-hexenyl acetate emitted by *T. urticae*-damaged lima bean (octanal was in the same peak), it was evident that this compound was completely destroyed at 120 nl l^{-1}.

Despite the evidence that the herbivore-induced terpenes and the GLVs were affected by increased O_3, *C. plutellae* females and *P. persimilis* were able to discriminate between the odor of intact plants and plants damaged by their respective host and prey. In the case of damaged cabbage in our system, O_3 efficiently degraded the herbivore-induced terpenes and the GLVs. Mattiacci et al. (1994) suggested that the low increases in terpenes compared

to the higher increases of other compounds after herbivore attack may reduce their relative contribution to the headspace blend of the related species *B. oleracea* var. *gemmifera*. Therefore, our results support the possibility that compounds other than terpenes are playing an olfactory role in tritrophic interactions and that these are plant-derived VOCs that are not rapidly reacting with O_3 or are products resulting from the reaction of O_3 with emitted compounds.

Scascighini et al. (2005) found the occurrence of a cineole, two GLVs, and a nitrile, benzyl cyanide, during the 5 hr after the onset of *P. brassicae* feeding on Brussels sprouts. In their study, the response of *C. glomerata* females increased steadily during the first 3 hr after the initiation of feeding. 1,8-Cineole was also emitted in the constitutive blend of cabbage plants and, therefore, its contribution to the active attractive blend might be low. The nitrile, benzyl cyanide, evokes a strong electrophysiological response in *C. glomerata* and *C. rubecula* (Smid et al., 2002). This prompted us to estimate the emissions of benzyl cyanide in our samples and its reactivity with O_3. This compound was emitted only by the herbivore-damaged cabbage plants, and its presence was clearly evident at 120 nl l^{-1} O_3. Our quantity estimates of benzyl cyanide in the samples showed that it remained unaffected by O_3. It has been suggested that specialized parasitoids of herbivores feeding on plants in the Brassicaceae such as *C. rubecula* may at any stage of the host location process use sulfur and nitrogen–sulfur-containing compounds, which are characteristic infochemicals for this plant family (Agelopoulos and Keller, 1994; Potting et al., 1999). This has been supported by no-choice and choice tests in wind tunnel experiments, which suggested that the volatiles emitted by insect-damaged cabbage plants attract *C. plutellae* regardless of the herbivore (Cameron and Walker, 1997) although *C. plutellae* showed significant preference for *P. xylostella*-damaged plants over artificially damaged and *P. rapae*-damaged plants (Shiojiri et al., 2001).

In contrast to *P. xylostella*, an oligophagous herbivore, *T. urticae* feeds on a wide range of plant species, thus eliciting the emission of different VOC blends (Van den Boom et al., 2004). However, the attraction of *P. persimilis* by *T. urticae*-damaged plants has been shown in different plant species belonging to different plant families (Dicke et al., 1998). We hypothesized that a predator in a less-specialized tritrophic system may rely more on the induced terpenes. Our results did not support this hypothesis; however, they support the observations of De Boer et al. (2004), who found that *P. persimilis* does not show preference when given the choice between synthetic forms of DMNT or TMTT and clean air.

In addition to emitting inducible terpenes, *T. urticae*-damaged plants emitted MeSA and 2-butanone. These are known to elicit a behavioral response in predatory mites (De Boer et al., 2004). It is interesting to note that there was significantly more MeSA at elevated O_3 levels. We do not expect that the increased quantities in the headspace of damaged plants are a consequence of the O_3 exposure because this compound was not observed in the VOC profile of intact plants at any O_3 concentration; however, this suggests a synergistic effect of O_3 and feeding damage. MeSA is a semi-VOC, and its lifetime with respect to oxidation by high concentrations of O_3 has been estimated at approximately 4 yr (Canosa-Mas et al., 2002). MeSA has been shown to be an important synomone (i.e., infochemical benefiting both the emitter and the receiver) for *P. persimilis*. It is not only attractive to this species when confronted alone opposite clean air in dual-choice tests but it is as attractive as *T. urticae*-damaged plants. Moreover, the addition of MeSA can make *Spodoptera exigua*-damaged plants attractive to predatory mites (De Boer et al., 2004) although it is not a compound induced exclusively by *T. urticae* feeding (see De Boer and Dicke, 2005 for a review). The other induced non-terpene compound was 2-butanone. It has been shown to be preferred over clean air in dual-choice tests (De Boer et al., 2004). The quantification of this

ketone reveals that it was not affected by increased O_3, suggesting that it may also enable discrimination by the predatory mites.

Plant–arthropod interactions mediated by plant volatiles are complex. In this study, we present some data that show that not only the decomposition of known herbivore-induced signaling compounds at elevated O_3 levels but also that the plant VOC emissions still provide reliable olfactory cues for natural enemies in two different tritrophic systems. This has not been demonstrated previously. Herbivore-induced blends consist of a series of compounds that vary in their reactivity in the atmosphere. Natural enemies might have adapted to use chemically stable compounds, such as benzyl cyanide or MeSA, which may be detected at long distances from the attacked plant. Other compounds with a long atmospheric lifetime, and released by herbivore-injured leaves, such as methanol (Atkinson and Arey, 2003; Peñuelas et al., 2005; Von Dahl et al., 2006), might also play roles as long-distance cues. Terpenes in the boundary layer of the plant might contribute in further steps of the foraging process. Some compounds that decreased below the detection limits of the GC-MS may still have played a role in the present study because insects have highly sensitive chemoreceptors (Schoonhoven et al., 2006). However, terpenes are highly reactive compounds that under natural conditions may be readily degraded not only by O_3 but also by OH and NO_3 (Atkinson and Arey, 2003). The oxidation of terpenes results in a series of products of the carbonyl family (Calogirou et al., 1999). It is also possible that natural enemies may have learned to recognize products of the reactions that are more stable and abundant in nature. The identification of such compounds is not easily achieved, and their role in chemical communication remains unknown. It would be interesting to address the preferences of natural enemies for herbivore-induced volatiles in O_3-free and in O_3-enriched natural environments to better understand the role of reactive compounds as olfactory cues. Natural enemies, of course, do not have the possibility of choosing an O_3-free environment during O_3 episodes in nature.

Acknowledgments The authors thank Timo Oksanen for the assistance in the development of the setup used in the experiment. D.P., J.B., and J.K.H. are supported by ISONET, MRTN-CT-2003-504720, and J.K. H. and A-M.N. by the Academy of Finland (ESGEMO programme, decision no. 105209).

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J Chem Ecol (2007) 33:695–710
DOI 10.1007/s10886-007-9260-y

Response of the Aphid Parasitoid *Aphidius funebris* to Volatiles from Undamaged and Aphid-infested *Centaurea nigra*

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Received: 17 August 2006 / Accepted: 29 January 2007 /
Published online: 2 March 2007
© Springer Science + Business Media, LLC 2007

Abstract Two issues have hindered the understanding of the ecology and evolution of volatile-mediated tritrophic interactions: few studies have addressed noncrop systems; and few statistical techniques have been applied that are suitable for the analysis of complex volatile blends. In this paper, we addressed both of these issues by studying the noncrop system involving the plant *Centaurea nigra*, the specialist aphid *Uroleucon jaceae*, and the parasitoid *Aphidius funebris*. In a Y-tube olfactometer, *A. funebris* was attracted to the odor from undamaged *C. nigra*, but preferred the plant–host complex (PHC) after 3 d of feeding by 200 *U. jaceae* over the undamaged plant, but not after three or 5 d of feeding by 50 *U. jaceae*. When aphids were removed, the initial preference for the damaged plant remained, but the final preference was not greater than for the undamaged plant. No qualitative differences were detected between the headspaces of *C. nigra* and the *C. nigra–U. jaceae* PHC. For quantitative analysis, we used a compositional approach, which treats each compound produced as part of a blend, and not as a compound released in isolation, thus allowing analysis of the relative contribution of each compound to the blend as a whole. With this approach, subtle increases and decreases of some green leaf volatiles and monoterpenoids on the third day of aphid infestation were detected. Mechanically damaged *C. nigra* had a volatile profile that differed from undamaged *C. nigra* and the PHC. One and 10 ng of (*Z*)-3-hexenyl acetate, and 10 or 100 ng of 6-methyl-5-hepten-2-one were attractive to the parasitoid when placed in solution on filter paper. *A. funebris* appears to be using a combination of chemical cues to locate host-infested plants.

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Keywords Tritrophic interactions · Multitrophic interactions · Induced responses · Semiochemicals · Plant volatiles · *Uroleucon jaceae* · Green leaf volatiles · Terpenoids · Indirect effects · Foraging behavior · Olfactometer · Compositional analysis

Introduction

Many parasitoids respond to plant odors (Turlings et al., 1991; Powell et al., 1998), and many can also distinguish undamaged from host-infested or previously host-damaged plants (Guerrieri et al., 1999; Lo Pinto et al., 2004; Moraes et al., 2005). Changes in volatile profiles that lead to increased parasitoid attraction have been characterized in many plant species (De Moraes et al., 1998; Röse et al., 1998; Bukovinszky et al., 2005), and it is apparent that volatiles released from storage immediately after damage, as well as those resulting from induction of biosynthetic pathways after herbivory, are important for attraction of parasitoids (Mattiacci et al., 2001; Hoballah and Turlings, 2005). Some responses are host-herbivore specific (Du et al., 1996; De Moraes et al., 1998; Shiojiri et al., 2001; Moraes et al., 2005), and attempts to characterize individual compounds that confer specificity to parasitoid responses have revealed 6-methyl-5-hepten-2-one as important as a specific indicator of the presence of *Acyrthosiphon pisum* for the parasitoid *Aphidius ervi* (Du et al., 1998), and the green-leaf volatiles (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate for the parasitoid *Cotesia glomerata* (Shiojiri et al., 2006). However, this specificity is not universal (e.g., Shiojiri et al., 2001; van Poecke et al., 2003).

Although the effects described above have been found for a wide range of parasitoids of pest insects, there is little information on the occurrence of plant volatile-mediated effects in noncrop systems. In noncrop systems, plants have not been modified by artificial selection, which could have had effects on plant metabolism that affect their volatile profiles. There is large variability in volatile production among closely related species in the maize genus *Zea* (Gouinguené et al., 2001) as well as among maize (*Z. mays*) cultivars (Turlings et al., 1998), and some domesticated species have less attractive blends than wild relatives (Bukovinszky et al., 2005). This indicates that artificial selection has led to changes in the metabolic pathways involved in the production of plant volatiles, and genetic uniformity in crops is likely to result in the loss of variability in volatile production. Thus, the identity and amount of volatiles released is likely to vary less among individuals in crop plants compared to noncrop plants. Crops come from a reduced subset of plants, mainly fast growing annual ruderals from early successional habitats (Feeny, 1976), so generalizations drawn from these studies might not always apply to natural ecosystems, for which noncrop plants need to be studied.

The aim of this study was: (a) to elucidate the olfactory responses of the parasitoid *Aphidius funebris* Mackauer (Hymenoptera, Braconidae) to the plant *Centaurea nigra* (L.) (Asteraceae, Cardueae) when it is undamaged, and when it is infested by the aphid *Uroleucon jaceae* (L.) (Aphididae, Aphidinae); (b) to determine the changes in *C. nigra* volatile chemistry that occur after attack by *U. jaceae* that might lead to increased attraction of *A. funebris* by using a compositional approach; and (c) to determine the importance of (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one as attractants of *A. funebris* because these compounds have electrophysiological activity in *A. ervi* (Du et al., 1998). This system provides a good contrast to crop systems studied because *C. nigra* is a perennial plant, which spends most of the year as a rosette and then bolts for flowering in early summer, and the aphid *U. jaceae* is highly specialized on *Centaurea* species, unlike most pest aphids

of herbaceous crops, which tend to be more polyphagous than their nonpest relatives (Blackman and Eastop, 2000). *A. funebris* is a specialist on *Uroleucon* spp., and attacks its hosts from outside the colony (Völkl et al., 1995), with greater success when attacking instars L2, L3, and L4, as compared to L1 and adults (Weisser, 1995).

Methods and Materials

Plants *Centaurea nigra* seeds were purchased from Herbiseed (Twyford, UK) and Emorsgate Wild Seeds (Norfolk, UK). They were germinated on damp paper, and plants were grown in a glasshouse under a 16:8 light:dark (L:D) regime and were aged between 35 and 45 d old, but of similar size (6–8 leaf stage) when used in experiments. None of the plants used had flowered.

Insect Rearing and Preparation for Bioassays *Uroleucon jaceae* were collected from the field at Rothamsted Farm, Herts, UK, between May and July 2002, and reared at 18°C and 16:8 L:D on *C. nigra*.

Aphidius funebris cultures were set up on *U. jaceae* feeding on *C. nigra* from mummies collected during June 2002 from *U. jaceae* (also feeding on *C. nigra*) in fields on Rothamsted Farm, Herts, UK, at 18°C and 16:8 L:D. Mummies were removed from culture plants and placed in an emergence cage. On the day of the bioassay, adult females were collected in glass vials and placed in the bioassay room to acclimatize for 1 hr. All females were less than 3 d old and were assumed to have mated before collection.

Y-tube Olfactometer Bioassays A glass Y-tube of 1 cm internal diameter, a 7 cm "trunk" and 5 cm arms was used as the bioassay arena. A pump provided an air stream that was passed through an activated charcoal filter and a flow meter before being split in two. Each stream was passed through a 5-l glass vessel containing an odor source and then through a flow meter set at 400 ml/min before entering an arm of the Y-tube. All connections between the parts described were 1 mm ID Teflon (PTFE) tubing. Twenty-four hours before each bioassay, the Y-tube, glass vessels, Teflon tubing, and glass fittings were all washed and rinsed with acetone and placed in an oven at 160°C.

All bioassays were carried out at 20°C on a white Formica bench under artificial lighting. On each arm of the Y-tube, a *choice line* was drawn halfway up the arm prior to the start of the bioassay. During the bioassay, each parasitoid was introduced individually into the base of the Y-tube. A first choice was recorded if the parasitoid crossed one of the choice lines drawn on the arms. A final choice was recorded if either the parasitoid spent more than 15 sec in motion beyond the choice line without crossing back out again, or if the parasitoid was not continuously in motion, if it spent 1 min beyond the choice line without crossing back out. To control for directional bias the arms of the Y-tube through which the odor sources were presented were swapped after every three parasitoids tested. Each parasitoid was used only once.

For testing the response of *A. funebris* to undamaged *C. nigra*, a single potted plant was taken directly from the glasshouse and tested against a pot filled with soil only. To test the response of *A. funebris* to the plant–host complex (PHC) relative to an undamaged plant, a set number of aphids of mixed ages (initial aphid density) were placed on a plant 3 d before the bioassay. Both the infested plant and the undamaged control plant were individually placed in porous Cryovac bread packing bags, which prevent aphid escape but do not accumulate humidity and allow air exchange. They were left in an 18°C controlled temperature room at

16:8 L:D. The responses of *A. funebris* to the following choices were compared: (1) PHC (initial aphid density of 50) vs. undamaged plant; (2) PHC (initial aphid density of 200) vs. undamaged plant. Additionally, the response to the PHC (initial aphid density of 50) after 5 d of aphid feeding vs. an undamaged plant was tested. Finally, to confirm that any responses to the PHC were due to the plant and not to semiochemicals from the aphid, the response of *A. funebris* to aphid-damaged plants (ADP) was tested. To do this, *C. nigra* plants were treated as described above for (2), and immediately before the bioassay was started all aphids were removed, and every leaf was swabbed with warm distilled water to remove aphid traces. The plant was then rinsed with distilled water, and any excess water remaining was removed with clean tissue paper. The same process was carried out for the undamaged plant to control for any effect of the cleaning process. Each of the bioassays described above was carried out on several (2–6) different days. On each day, a single plant or combination of plants was used, and between 10 and 40 parasitoids were tested on any given day.

The dose responses of *A. funebris* to (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one were measured in the Y-tube olfactometer because the related parasitoid *A. ervi* had previously shown electrophysiological responses to these compounds (Du et al., 1998). Solutions were prepared from synthetic commercial (*Z*)-3-hexenyl acetate (Aldrich, Milwaukee, WI, USA) and 6-methyl-5-hepten-2-one (Aldrich, St. Louis, MO, USA). For the bioassays, either 10 μl of the bioassay solution or 10 μl hexane (control) were placed on a 7-cm (diameter) circular piece of clean filter paper at the bottom of each of two 500 ml glass vessels, which were left for 1 min before starting the bioassay to allow the solvent to evaporate. Throughout the bioassay, the arms delivering the test and control odors were alternated after every three parasitoids tested. The amounts of (*Z*)-3-hexenyl acetate tested were 0.1, 1, 10, and 100 ng. For 6-methyl-5-hepten-2-one the amounts tested were 1, 10, 100, and 1,000 ng.

Volatile Collection by Air Entrainment Super Q 80/100 mesh (Alltech, PA, USA) was used to adsorb the volatiles and was placed in small glass tubes secured with glass wool. The adsorbent was conditioned with hexane and diethyl ether, and heated at 135°C with a flow of nitrogen for 24 hr. Between uses, the adsorbent tubes were rinsed with 1 ml ether and heated (135°C) with a nitrogen flow for at least 3 hr.

Plant volatiles were collected by placing the aerial parts of the plant in a 2.2 l glass vessel, closed at the base with two metal plates. Because the plates do not seal the vessel, the soil in which the plant was growing was covered with aluminium foil to minimize volatiles from the soil seeping into the air entrainment vessel. The adsorbent tube was secured in one of the apertures at the top of the vessel, and through another aperture a Teflon tube carrying charcoal-filtered air was inserted into the vessel. Air was pumped into the vessels at a rate of 1.2 l/min and sucked through the adsorbent tube at 1 l/min for 24 hr. Before every air entrainment, all glass, metal and nondisposable Teflon material was washed, rinsed with acetone, and heated at 160°C for 24 hr. The adsorbed compounds were eluted with 500 μl of redistilled ether, and each sample was concentrated to 100 μl under a gentle flow of filtered nitrogen gas. Blanks were regularly carried out to test the purified air for impurities.

Air Entrainments Immediately before the start of the air entrainment, 200 *U. jaceae* of mixed ages were placed on one of the two plants (randomly selected), while the other plant was left undamaged. Fourteen replicate air entrainments were carried out over 5 d, pairing undamaged *C. nigra* with the PHC in a randomized block design. The adsorbent tube was changed every 24 hr to obtain daily profiles (ignoring variation in volatile production due to the light cycle).

Air entrainments with mechanically damaged *C. nigra* were carried out by cutting 1 cm off every leaf tip of a plant to determine the compounds released upon mechanical damage (not to mimic aphid damage). Six paired (undamaged and damaged) air entrainments were carried out for 3 d, with a sample taken every 24 hr.

Compound Identification Volatile samples were injected into a coupled gas chromatography–mass spectrometry (GC–MS) system for identification (Thermo-Finnigan MAT95XP magnetic sector mass spectrometer), equipped with an ion trap analyzer, which was directly coupled to a Finnigan Trace 2000 GC with a non-polar 50 m HP-1 column (methyl siloxane, 0.52 μm film thickness, 250 μm ID, J&W Scientific, Folsom, CA, USA) with helium as carrier gas. Ionization was by electron impact (70 eV, source temperature 200°C). Two microliters were injected for each sample in a temperature ramp program (30°C/5 min, 5°C/min to 250°C/21 min).

Mass spectra for the compounds were compared against a NIST library. A tentative identification was made if the library match was greater than 75% and the KI matched when compared to a database. GC analyses (Hewlett Packard 6980) were carried out by cool-on-column injection of standards and samples in temperature ramps in a polar 25 m DB-Wax column (polyethylene glycol, 0.50 μm film thickness, 320 μm internal diameter, J&W Scientific, Folsom, CA, USA; 4.00 psi, 30°C/0.10 min, 2°C/min to 100°C/0.10 min, 3°C/min to 150°C/0.10 min, 10°C/min to 250°C/8 min), and a nonpolar HP-1 column (dimensions as above; 10.00 psi, 30°C/0.10 min, 5°C/min to 150°C/0.10 min, 10°C/min to 230°C/20 min), with an inlet temperature of 30°C and hydrogen as carrier gas. Detection was by a flame ionization detector (FID) at 250°C. Fragmentation of the compounds from the sample was compared to the fragmentation of standards when available. Peak enhancement coinjection was carried out with authentic standards to confirm the identities of compounds. The sources of authentic compounds were as follows: (*Z*)-3-hexenyl acetate (98+%): Aldrich, Milwaukee, WI, USA; alkane series, hexanal (98%), butyl acetate (99+%), (*E*)-2-hexenal (98%), 6-methyl-5-hepten-2-one (99%), α-pinene (98%), (1*S*)-(−)-β-pinene (99%), 3-carene (90%), (+)-longifolene (98+%): Aldrich, St. Louis, MO, USA; (−)-camphene (85%), sabinene (98.5%), myrcene (90%), (*R*)-(−)-α-phellandrene (50%), (*R*)-(+)-limonene (96%), γ-terpinene (97%), (+,−)-camphor (95%), (+)-cyclosativene, (−)-α-copaene (90%), β-caryophyllene (98.5%), α-humulene (98%): Fluka, Buchs., Switzerland; (*E,E*)-α-farnesene and (*E*)-β-farnesene: provided by J. Aldrich (USDA, Beltsville, MD, USA).

Volatile Quantification Volatiles from undamaged *C. nigra* and the PHC were quantified by using GC analysis with heneicosane (25 ng/μl) as the internal standard (IS). Samples were injected into an HP-1 column as described above. The total amount of each compound released in the 24 hr of each air entrainment was calculated.

Design and Statistical Analyses For the Y-tube bioassay, data analyses were carried out for first and final choices of the parasitoid. The *responsiveness* (*r*) refers to the percentage of parasitoids making a choice for one of the odors in that bioassay and only these parasitoids were used in the analyses.

The first and final choices of all responding insects were summarized into daily 2×2 contingency tables of counts classified by the arm chosen by the parasitoid (left or right) and the side the test odor was presented (left or right arm). The treatment effect on each day is hence represented by the interaction (diagonal) in each table. The counts from all days for first or final choice were then analyzed by using a generalized linear model (GLM) with Poisson errors and logarithm link function. Main effects of day (replicates) were removed

before fitting other terms. The significance of the treatment effect was assessed by comparing the deviance for the interaction term against tabulated χ^2 values with 1 *df*.

Three approaches were used for the analysis of volatile compounds. First, the mean total amounts of volatiles (transformed to natural logarithms) produced in each 24 hr period were compared between plant treatments (either aphid-infected or undamaged) by using a split-plot repeated measures analysis of variance (ANOVA), as implemented in the AREPMEASUREMENTS procedure of GenStat (Payne et al., 2005). Blocks represented groups of entrainments carried out simultaneously, and repeated measures over time were treated as split-plots within each entrainment. Degrees of freedom in the lowest (time) stratum were corrected by using Greenhouse and Geisser's (1959) epsilon statistic to account for patterns of unequal correlation among days (a violation of ANOVA split-plot assumptions). Confidence limits (95%) for means were calculated on the logarithmic scale and back-transformed for presentation.

To assess the changes in different classes of compounds, each of the compounds identified and quantified was allocated to one of five classes: (1) aldehydes, ketones, and esters (AKE); (2) hydrocarbons; (3) monoterpenoids; (4) sesquiterpenoids; and (5) *unknowns*. For each class separately, the total amount of volatiles summed over all allocated compounds was converted to logarithms and analyzed by using repeated measures ANOVA as described above for the 5 d of entrainment. Confidence limits (95%) were calculated on the logarithmic scale and back-transformed for presentation.

Finally, because the volatiles emitted are part of a blend of compounds produced by one plant, and hence are not independent, multivariate log-ratio analyses (Aitchison, 1986) were used to analyze amounts of all compounds simultaneously, both at the individual compound level within each of the five classes separately and for the class totals. This analysis considers the contribution of each component relative to the rest of the components. Thus, it is suitable for comparing multivariate data that are intrinsically interrelated, as is the case for volatile compounds produced by plants. At the compound level the data for each replicate were first standardized by expressing the amount of each compound produced as a proportion of the total amount of volatiles in that class. Similarly, at the class level, the total amount of volatiles of each type was expressed as a proportion of the total volatiles produced. One component (compound or class) was used as a baseline (see below), and the amounts of all other components were expressed as a ratio of the amount of the baseline components. These ratios were then logged (natural) and analyzed with multivariate analysis of variance (MANOVA). If one or more components were absent for any plant, a proportional correction was applied (see Aitchison, 1986).

When analyzed at the class level, the baseline was the *unknowns* class. This allowed an assessment of the differences in contribution of each class of compounds to the blend in the undamaged *C. nigra* treatment compared to the PHC treatment. When analyzing at the compound level within each class separately, the following compounds were used as the baseline: hexanal in the AKE class; octane in the hydrocarbon class; α-pinene in the monoterpenoid class; and cyclosativene in the sesquiterpenoid class.

Within each MANOVA, the overall effect of treatment was assessed by using an *F* test, but, to determine which variables contributed to any observed treatment differences, a canonical variate analysis (CVA) was also used to search for a linear combination of the variables that best discriminates between the treatments. With two treatments, there is only one possible canonical variate, and the CVA loadings are one dimensional, and represent the contribution of each particular log-ratio variable to the discrimination between treatments. Biplots of the CVA scores (for individual plants) and loadings enabled visual inspection of

which variables contributed to the separation of the undamaged *C. nigra* and PHC treatments.

Results

Full Plant Bioassays Aphidius funebris preferred the odor of *C. nigra* over clean air on first and final choice (Fig. 1a, b). The parasitoid showed no significant preference for the 3-d-50-aphid plant–host complex (PHC) odor, or for the 5-d-50-aphid PHC odor over that of undamaged *C. nigra*. However, the 3-d-200-aphid PHC odor was preferred over undamaged *C. nigra* by *A. funebris* on both first and final choice. Also, the aphid-damaged plant (ADP) odor was preferred over that from undamaged *C. nigra* on first choice, although the final choice preference was not statistically significant (Fig. 1a, b).

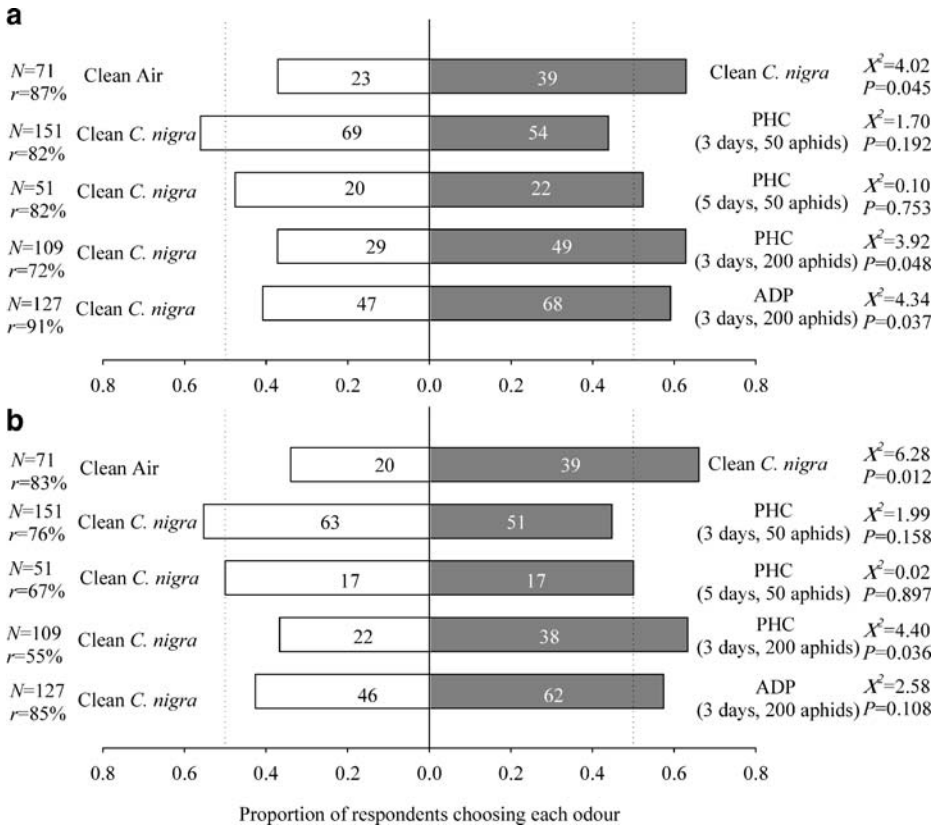


Fig. 1 First (**a**) and final (**b**) responses of *A. funebris* to *C. nigra* odors in a Y-tube olfactometer. Clean *C. nigra*=undamaged plant. For the plant–host complexes (PHC) and the aphid-damaged plant (ADP), the time they were infested before the bioassay and the initial number of aphids are indicated in brackets. *N* is the total number of parasitoids tested and *r* is the responsiveness. Within the bar is the number choosing each odor. The *horizontal axis* gives the proportion of responding insects that chose each odor, and the *dotted line* indicates no preference (50% responses in each arm). X^2 values are obtained from the change in deviance of the GLM interaction term upon deletion from the model (see text for detail)

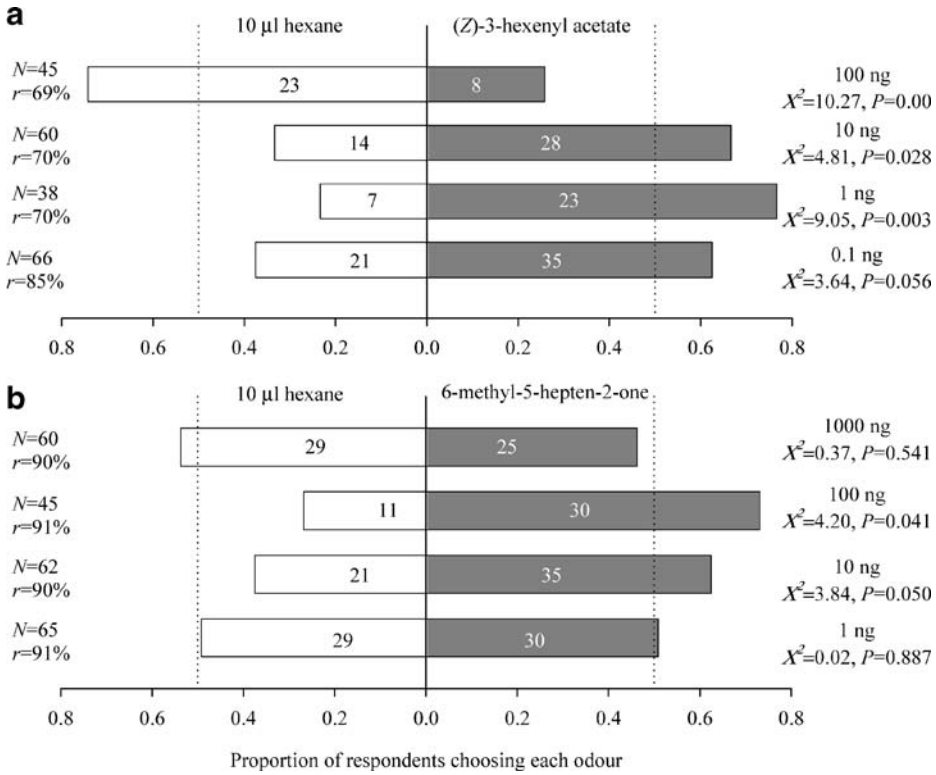


Fig. 2 Dose response of *A. funebris* to (**a**) (*Z*)-3-hexenyl acetate and (**b**) 6-methyl-5-hepten-2-one in a Y-tube olfactometer. *N* is the total number of parasitoids tested and *r* is the responsiveness and within the bar are the number choosing each odor. The *horizontal axis* gives the proportion of responding insects that chose each odor, and the *dotted line* indicates no preference. X^2 values are obtained from the change in deviance of the GLM interaction term upon deletion from the model (see text for detail)

Dose Response Curves Only the first choice responses are presented here. *Aphidius funebris* significantly avoided (*Z*)-3-hexenyl acetate at the highest dose of 100 ng, but showed a positive response to 10 and 1 ng of (*Z*)-3-hexenyl acetate. The response to the lowest dose of 0.1 ng also appeared to be positive, but was not statistically significant (Fig. 2a). The parasitoid showed a positive response to 6-methyl-5-hepten-2-one at 100 and 10 ng doses, but not at the highest (1,000 ng) and lowest (1 ng) doses (Fig. 2b).

Volatile Profiles A total of 40 compounds were identified (of which nine tentatively) (Table 1). The volatile profile of *C. nigra* and the *C. nigra–U. jaceae* PHC did not differ qualitatively.

Repeated Measures Analyses of Volatile Profiles The total amount of volatiles produced in 24 hr was neither affected by aphid infestation ($F_{1,14}$=0.40, P=0.535) nor by time ($F_{2.93,74.65}$= 1.96, P=0.128), and the effect of treatment did not change with time (no significant interaction: $F_{2.93,74.65}$=1.55, P=0.210) (Table 1).

Of the four classes of identified compounds, hydrocarbons, AKEs (aldehydes, ketones, and esters), monoterpenoids, and sesquiterpenoids, none was affected by treatment (P>

Table 1 Compounds identified from *C. nigra* and volatile amounts produced by undamaged *C. nigra* and the *C. nigra–U. jaceae* plant–host complex (PHC)

Compounds[a]	KI		Mean (±SE) Ln Amount Released (In[ng/24 hr])									
	HP-1	DB-Wax	Undamaged *C. nigra*					*C. nigra–U. jaceae* PHC				
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Hydrocarbons												
1. Octane	800	800	2.43±0.61	2.56±0.52	2.66±52	2.54±0.56	2.72±0.60	1.78±0.44	2.98±0.68	3.08±0.610	3.14±0.65	3.23±0.61
2. Nonane	900	900	1.79±0.44	1.63±0.38	1.27±0.40	1.62±0.41	1.39±0.41	1.38±0.41	2.05±0.38	1.97±0.39	1.26±0.45	1.94±0.38
3. Decane	1,000	1,000	2.10±0.49	2.28±0.47	1.90±0.47	2.17±0.56	1.90±0.47	2.01±0.51	2.39±0.50	2.32±0.51	2.07±0.50	2.59±0.43
4. Undecane	1,100	1,100	2.31±0.47	2.23±0.47	1.59±0.50	2.07±0.48	2.17±0.49	2.03±0.51	2.47±0.51	2.68±0.48	2.18±0.51	2.33±0.51
5. Dodecane	1,200	1,200	2.23±0.47	2.15±0.51	1.51±0.52	2.17±0.49	2.09±0.48	2.10±0.51	2.35±0.52	2.73±0.46	1.96±0.52	2.26±0.50
6. Tridecane	1,300	1,300	2.05±0.50	1.83±0.50	1.38±0.49	2.05±0.53	1.82±0.48	1.94±0.50	2.22±0.48	2.56±0.45	1.86±0.49	2.20±0.45
7. Tetradecane	1,400	1,400	2.58±0.47	2.29±0.50	1.99±0.50	2.27±0.54	2.69±0.45	2.37±0.50	2.54±0.45	2.39±0.48	2.05±0.51	2.24±0.50
8. 1-Pentadecene (T)	1,491	–[a]	Nq	0.56±0.39	Nq	Nq	Nq	1.31±0.54	Nq	1.57±0.65	1.11±0.59	0.78±0.53
9. Pentadecane	1,500	1,500	2.77±0.36	2.60±0.37	2.29±0.42	2.45±0.47	2.58±0.48	1.95±0.47	3.16±0.33	2.82±0.40	1.91±0.51	2.36±0.43
10. 1-Hexadecene (T)	1,591	–[a]	2.24±0.59	Nq	Nq	Nq	Nq	Nq	2.19±0.60	0.88±0.46	Nq	2.24±0.44
11. Hexadecane	1,600	1,600	1.60±0.29	1.55±0.31	1.02±0.32	1.38±0.31	1.14±0.34	1.65±0.33	1.80±0.28	1.17±0.34	0.99±0.35	1.09±0.34
12. 3-Heptadecene (T)	1,691	–[a]	1.41±0.46	1.93±0.55	1.74±0.51	1.92±0.50	1.63±0.47	2.13±0.26	2.26±0.48	2.16±0.39	1.92±0.50	
Aldehydes												
13. Hexanal	775	1,054	1.92±0.48	1.78±0.48	1.22±0.43	1.59±0.39	1.86±0.36	1.46±0.40	2.17±0.44	2.36±0.52	2.36±0.46	2.28±0.41
14. (*E*)-2-Hexenal	827	1,179	1.97±0.41	2.22±0.34	1.26±0.36	1.38±0.32	1.23±0.28	1.21±0.36	2.10±0.47	1.85±0.37	1.49±0.30	1.48±0.27
Ketones												
15. 6-Methyl-5-hepten-2-one	965	1,298	1.34±0.47	1.14±0.50	0.93±0.42	0.68±0.37	0.97±0.37	1.46±0.45	1.79±0.50	1.63±0.54	1.28±0.52	1.43±0.54
Esters												
16. Butyl acetate	797	–[a]	Nq	0.53±0.31	0.25±0.17	0.56±0.32	0.65±0.32	Nq	0.59±0.27	0.99±0.37	0.93±0.35	0.51±0.24
17. (*Z*)-3-Hexenyl acetate	988	1,283	5.97±0.63	2.78±0.84	2.84±0.68	1.65±0.55	1.84±0.49	4.21±0.90	4.29±0.95	3.98±0.78	2.78±0.75	2.36±0.69
Monoterpenoids												
18. Tricyclene (T)	921	–[a]	1.15±0.37	0.84±0.38	0.67±0.34	0.59±0.31	0.59±0.32	0.89±0.32	0.72±0.40	1.27±0.43	0.71±0.39	0.87±0.39
19. α-Thujene (T)	928	–[a]	0.75±0.33	0.59±0.33	0.67±0.33	0.81±0.56	0.49±0.27	0.62±0.32	0.83±0.38	1.42±0.51	0.75±0.46	1.41±0.49
20. α-Pinene	931	1,006	6.24±0.49	6.21±0.47	5.97±0.53	5.63±0.58	5.92±0.52	5.65±0.67	6.44±0.49	6.67±0.43	6.41±0.38	6.45±0.45
21. α-Fenchene (T)	944	–[a]	2.49±0.43	1.75±0.56	1.89±0.54	1.84±0.51	2.08±0.55	2.05±0.53	2.59±0.56	2.74±0.50	2.19±0.54	2.54±0.49
22. Camphene	944	1,047	2.82±0.46	3.09±0.41	2.71±0.49	2.69±0.47	2.65±0.51	2.58±0.48	3.00±0.52	3.09±0.48	2.86±0.43	3.08±0.40
23. Sabinene	967	1,099	1.93±0.49	1.32±0.50	1.86±0.53	1.83±0.52	1.69±0.49	1.81±0.54	1.27±0.47	2.05±0.51	1.82±0.53	1.66±0.48
24. β-Pinene	970	1,085	3.24±0.48	3.21±0.45	2.90±0.54	2.83±0.48	3.08±0.47	3.02±0.53	3.30±0.51	3.67±0.45	3.33±0.44	3.55±0.45
25. Myrcene	983	1,145	2.68±0.52	1.50±0.50	1.22±0.49	1.22±0.49	1.48±0.50	2.41±0.56	1.64±0.55	2.23±0.46	1.64±0.49	2.02±0.50

Table 1 (continued)

Compounds[a]	KI		Mean (±SE) Ln Amount Released (ln[ng/24 hr])									
	HP-1	DB-Wax	Undamaged *C. nigra*					*C. nigra–U. jaceae* PHC				
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
26. α-Phellandrene	998	1,145	0.39±0.27	0.40±0.27	0.43±0.29	Nq	Nq	Nq	Nq	0.81±0.44	Nq	0.46±0.31
27. 3-Carene	1,003	1,128	5.42±0.36	5.34±0.40	5.07±0.42	4.56±0.59	4.66±0.63	4.76±0.57	5.39±0.48	5.32±0.59	5.21±0.47	5.34±0.48
28. Limonene	1,021	1,176	4.66±0.56	4.46±0.56	4.18±0.51	3.81±0.62	3.60±0.68	3.93±0.65	4.96±0.59	4.60±0.57	4.40±0.46	4.18±0.54
29. γ-Terpinene	1,050	1,222	0.39±0.27	0.62±0.34	0.43±0.29	Nq	0.39±0.28	0.35±0.22	0.45±0.32	0.58±0.40	0.63±0.43	0.86±0.35
30. Camphor	1,118	1,469	Nq	0.28±0.19	0.42±0.31	0.40±0.28	Nq	Nq	0.51±0.36	0.68±0.37	0.40±0.28	0.39±0.20
Sesquiterpenoids												
31. Cyclosativene	1,372	1,464	1.20±0.31	1.45±0.35	1.58±0.37	1.30±0.31	1.54±0.38	1.05±0.31	1.64±0.39	1.87±0.38	1.30±0.39	1.52±0.38
32. α-Copaene	1,379	1,478	1.35±0.35	1.30±0.30	1.43±0.27	1.74±0.28	1.94±0.26	1.14±0.38	1.41±0.36	2.10±0.36	1.92±0.37	1.95±0.35
33. β-Cubebene (T)	1,394	–[a]	1.24±0.42	2.28±0.46	1.53±0.45	1.61±0.43	1.98±0.43	1.78±0.43	1.87±0.46	2.11±0.43	2.04±0.48	2.08±0.42
34. Longifolene	1,406	1,543	1.10±0.37	1.17±0.32	1.11±0.25	1.35±0.35	1.55±0.34	0.83±0.28	1.31±0.38	1.68±0.40	2.06±0.45	1.79±0.31
35. β-Caryophyllene	1,420	1,570	0.51±0.24	0.36±0.20	0.54±0.19	0.91±0.28	1.07±0.25	0.74±0.26	0.86±0.31	1.13±0.34	1.16±0.29	1.24±0.31
36. α-Bergamotene (T)	1,439	–[a]	0.89±0.33	0.45±0.26	0.72±0.31	0.41±0.21	1.20±0.33	0.79±0.31	1.23±0.37	0.89±0.36	1.10±0.30	1.03±0.27
37. (*E*)-β-Farnesene	1,449	1,639	0.79±0.29	0.61±0.27	0.95±0.30	0.98±0.26	1.27±0.27	2.07±0.38	2.34±0.32	2.42±0.33	2.45±0.28	2.44±0.36
38. α-Humulene	1,455	1,698	2.52±0.54	1.51±0.51	1.89±0.51	1.93±0.46	0.93±0.35	1.14±0.30	1.91±0.56	2.17±0.40	1.62±0.38	1.32±0.32
39. Germacrene D (T)	1,488	–[a]	0.52±0.27	0.31±0.19	0.16±0.11	0.48±0.19	1.01±0.35	0.64±0.23	0.31±0.15	1.06±0.33	1.37±0.36	1.46±0.39
40. (*E,E*)-α-Farnesene	1,495	1,718	0.83±0.38	1.00±0.41	1.01±0.40	0.88±0.30	1.36±0.42	1.58±0.47	0.43±0.30	1.22±0.41	1.12±0.38	1.15±0.41
Total amount			8.98±0.16	8.81±0.16	8.62±0.16	8.62±0.16	8.59±0.16	8.80±0.16	8.97±0.16	8.93±0.16	8.73±0.16	8.78±0.16

KI values were calculated on HP-1 and DB-Wax columns from standards (see text for detail). The amounts presented are the mean and SE of the natural logarithm of the amount produced in 24 hr of entrainment (N=14), calculated relative to the area of the internal standard.

(*T*) indicates tentative identifications where no peak enhancement was carried out with authentic standards; *Nq* not quantifiable

[a] KI calculated from assumed compound (from fragmentation pattern) on HP-1 column in the GC-MS and no standard available for calculation on DB-Wax.

0.05), and only the AKEs showed an effect of time ($F_{2.27,57.94}$=7.26, P<0.001) and a time by treatment interaction ($F_{2.27,57.94}$=3.88, P=0.022). For this group, there was a sharp decrease in the amount produced by undamaged *C. nigra* from the first to subsequent days, but the amount produced by the PHC increased (from a level lower than in the undamaged *C. nigra*) and then decreased.

Compositional Analyses of Volatile Profiles During the 1st d (0–24 hr) of entrainment, the contribution of the different classes to the volatile profiles of *C. nigra* plants differed between treatments ($F_{4,10}$=3.57, P=0.047). The AKE class (loading=0.42) was associated with undamaged *C. nigra*, while the sesquiterpenoids (loading=−0.42) were associated with the PHC, and these contributed to separating treatments more than the monoterpenoid or hydrocarbon classes (loadings=0.11 and 0.09, respectively) (Fig. 3a).

During the fourth day, there was a marginally nonsignificant effect of treatment ($F_{4,11}$= 3.24, P=0.055) on the contributions by the different classes of compounds, and the main contribution was by hydrocarbons (loading=0.79), which were associated with the undamaged *C. nigra*. This was opposed by the AKE class (loading=−0.62), which was associated with the PHC. The monoterpenoid and sesquiterpenoid classes were more associated with the PHC, but their loadings were lower (Fig. 3b). On the second day ($F_{4,10}$=1.71, P=0.224), third day ($F_{4,11}$=2.12, P=0.146), and fifth day ($F_{4,11}$=0.92, P=0.488), no difference between treatments were detected in the contribution of the four classes.

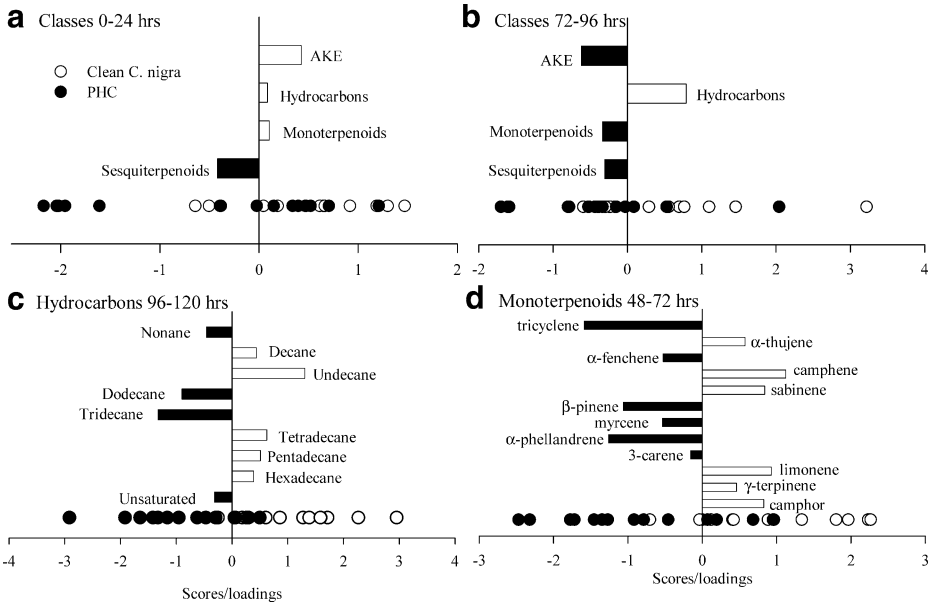


Fig. 3 Canonical variate analysis (CVA) biplots from the comparisons between undamaged (clean) *C. nigra* and the *C. nigra–U. jaceae* plant–host complex (PHC) air entrainments. **a** Compositional analysis for the classes of compounds (acetates, ketones, and esters (AKE), hydrocarbons, monoterpenoids, and sesquiterpenoids) on the first day (0–24 hr) of aphid damage. **b** Class compositional analysis for the fourth day (72–96 hr). **c** Hydrocarbon compositional analysis for the fifth day (96–120 hr). **d** Monoterpenoid compositional analysis for the third day (48–72 hr). The *points* represent the CVA scores for each individual replicate and the *bars* are the CVA loadings, indicating the contribution of each variable towards separating the treatments

Of the hydrocarbons listed in Table 1, the three unsaturated hydrocarbons (1-pentadecene, hexadecene, and 3-heptadecene) were grouped together for the analysis, and the others were included individually. Within this group, there was a marginal effect on the fifth day ($F_{9,6}$= 3.27, P=0.081), and the greatest contribution to separating the treatments came from tridecane, which was associated with the PHC, and undecane, which was associated with undamaged *C. nigra* (Fig. 3c). Dodecane was associated with the PHC. Days 1–4 revealed no differences between the treatments ($F_{9,5}$=0.34, P=0.926; $F_{9,5}$=0.41, P=0.883; $F_{9,3}$= 0.61, P=0.749; $F_{9,6}$=2.29, P=0.162, respectively).

The AKE class consisted of hexanal, (*E*)-2-hexenal, 6-methyl-5-hepten-2-one, butyl acetate, and (*Z*)-3-hexenyl acetate. The composition of the compounds within this class did not vary between the treatments on any of the days of entrainment (in order: $F_{5,8}$=1.18, P= 0.396; $F_{5,9}$=0.31, P=0.896; $F_{5,10}$=0.18, P=0.965; $F_{5,10}$=0.24, P=0.936; $F_{5,9}$=0.57, P= 0.721).

For the compounds within the monoterpenoid class, a marginally nonsignificant effect on the third day of entrainment ($F_{12,3}$=5.68, P=0.089) was observed. On this day, three compounds (tricyclene, β-pinene, and α-phellandrene) were strongly associated with the PHC, while four compounds (camphene, sabinene, limonene, and camphor) were associated with undamaged *C. nigra* (Fig. 3d).

Within the sesquiterpenoids, none of the compounds changed in its contribution to the volatile blend over the 5 d of entrainment (for the 5 d in order: $F_{10,3}$=1.48, P=0.414; $F_{10,4}$= 1.68, P=0.326; $F_{10,5}$=2.34, P=0.181; $F_{10,4}$=1.40, P=0.398; $F_{10,5}$=2.16, P=0.205).

Mechanical Damage Tests Changes due to mechanical damage trials were not quantified, but it led to an apparent increase in 1-pentadecene, 1-hexadecene, germacrene D, (*E,E*)-α-farnesene, and β-caryophyllene. This increase in volatiles occurred during the first 24 hr after damage, and then decreased back to the same levels as the undamaged plant on the second and third day of entrainment.

Discussion

We have demonstrated that the parasitoid *A. funebris* responds to odors from undamaged *C. nigra* and the *C. nigra–U. jaceae* PHC. A high initial aphid density is needed for increased parasitoid attraction, which contrasts with the *Vicia faba–A. pisum* system, where 40 aphids feeding for 3 d were enough to trigger changes in parasitoid behavior (Guerrieri et al., 1999). The species studied have important ecological differences compared to other plant–aphid systems studied in a tritrophic context. In particular, the plant is perennial and the aphid highly specialized, and such a plant-aphid relationship is likely to be tightly coupled. The aphid is likely to overcome host plant defenses efficiently, with minimal effects on plant physiology, leading to small changes in volatile chemistry. Herbivore-induced changes in plant volatile chemistry probably evolved primarily as a direct result of plant–aphid interactions, and predators and parasitoids subsequently evolved responses to the subtle changes in plant volatile profiles resulting from these interactions. This suggests that "plant signalling" was originally nonadaptive in the tritrophic context, and is a function acquired after the natural enemies evolved responses to plant volatiles (Bronstein and Barbosa, 2002).

Host plant specialization by aphids leads to morphological adaptation to those host plants (Moran, 1986), and this might be reflected in the adaptation of feeding structures to the host plant because there is within-species variation in the stylet movement in aphids (Bernays

and Funk, 2000). Such physical adaptation would be important in reducing defensive metabolic changes in the plant, and the stylet path is known to play a role in reducing plant responses to injury (Will and van Bel, 2006) via the stylet sheath, which might have a function in dampening the wound responses of the plant (Miles, 1999) and as a "plug" preventing the influx of calcium ions, which are important in activating defense proteins in the phloem (Tjallingii, 2006; Will and van Bel, 2006).

The lack of induced terpenoids observed in *C. nigra* could be due to low jasmonic acid (JA) levels, which could be a result of these reduced wound responses (Fäldt et al., 2003; Pauw and Memelink, 2004), but could also be due to inhibition by salicylic acid (SA)-dependent gene expression, which is activated by many phloem feeders, including several aphid species (Walling, 2000; Moran and Thompson, 2001; Martinez de Ilarduya et al., 2003). The interaction between JA and SA dependent responses can be antagonistic, though there are also cases of simultaneous activation of both defense pathways (reviewed by Bostock, 2005). This interaction can be important in determining the quantity and identity of induced volatiles, and can provide a mechanism of species-specific volatile emission (Kessler and Baldwin, 2002; Dicke et al., 2003; Heidel and Baldwin, 2004; Kaloshian and Walling, 2005).

The parasitoid showed a significant response to the ADP on first choice, but not on final choice. It is possible that the presence of aphids provides cues that reinforce its attraction to the plant. As it gets closer to the host it encounters and uses more reliable cues (Vet and Dicke, 1992), and if it fails to perceive these more specific cues, it might give up that particular search. It is likely that *A. funebris* uses a sequence of cues, each reinforcing the previous, to find its hosts (Vinson, 1976). The lack of qualitative changes in the volatile profile of *C. nigra* detectable by GC-FID suggests that quantitative changes in several compounds of different classes are responsible for attraction of *A. funebris* to aphid-infested plants, and this is likely due to the interactions between signalling pathways and aphid-specific responses discussed above. The small relative changes in "minor" and abundant monoterpenoids may serve as cues that *A. funebris* can exploit. In particular, the changes in relative contributions of tricyclene, β-pinene, myrcene, and α-phellandrene (greater relative presence after herbivory), and camphene, sabinene, limonene, and camphor (greater relative presence in undamaged plants) might be important. To determine this, electroantennography (EAG) and bioassays with different ratios of compounds are needed. Changes in volatile composition are often quantitative (e.g., Colazza et al., 2004b; Moraes et al., 2005), and use of ratios can provide specific information on herbivore presence and identity for some species of parasitoid (De Moraes et al., 1998; Smid et al., 2002). In individual parasitoid antennae, many different compounds show EAG activity (Du et al., 1998; Smid et al., 2002; Gouinguené et al., 2005). Specific types of damage are often needed for maximum attraction of parasitoids (Mattiacci et al., 2001; Colazza et al., 2004a; Hoballah and Turlings, 2005), and individual compounds with EAG activity are not always attractive in isolation (Ngi-Song et al., 2000), suggesting that the use of a combination of compounds might be a common strategy. *Aphidius funebris* therefore differs from *A. ervi*, which seems to use 6-methyl-5-hepten-2-one as a specific indicator of the presence of a suitable aphid (Du et al., 1998), and we agree with the conclusion by Bukovinszky et al. (2005) that subtle changes are responsible for attraction.

Aphidius funebris responds to (*Z*)-3-hexenyl acetate, a ubiquitous green leaf volatile. This has been shown for many parasitoid species (Takabayashi et al., 1991; Whitman and Eller, 1992; Röse et al., 1998; Hoballah and Turlings, 2005) and might provide a means of finding vegetation, but specificity is probably due to components present in relatively low amounts in the volatile blend (Gouinguené et al., 2005). The responses to cues of increasing reliability as

the parasitoid gets nearer to its host is likely to be highly influenced by heterogeneity at different spatial scales, many of which are absent in crop systems because the patch is usually a field of a single plant, highlighting the importance of studying noncrop systems.

The PHC showed an increased emission of sesquiterpenoids on the first day after aphid infestation (Fig. 3a). This is probably due to minor mechanical damage by the aphids because the mechanical damage tests showed an increase in several sesquiterpenoids during the first day. These compounds are probably released from storage upon damage, possibly due to the systemic JA-mediated wound response (Paré and Tumlinson, 1997), and probably indicate general damage.

This study has shown that semiochemical-mediated parasitoid attraction occurs in this noncrop system, and could help explain patterns of parasitism in the field because it has been demonstrated (Pareja, 2006) that individuals in larger colonies are at greater risk of parasitism than those in smaller colonies in the field (i.e., parasitism is directly density dependent). This suggests that response to volatiles could mediate processes such as density dependence that affect community structuring and population dynamics in the field, and are likely to be highly scale-dependent. This has been suggested in the past (Vet, 1996, 1999), but the studies that have demonstrated the potential for semiochemical interactions mediating host-parasitoid population and community processes are few (see e.g., Geervliet et al., 2000; Roßbach et al., 2005).

Effects in this system are subtle, and the parasitoid *A. funebris* is sensitive to minor changes in plant volatile profiles. The statistical technique we present for analyzing volatile data has a firm base outside ecology, but has never been utilized in chemical ecology. We believe that it is suitable for dealing with the complexity of volatile production by plants because it treats volatile compounds as parts of a blend, and not as unrelated compounds released in isolation, which makes intuitive sense when we consider the interrelated metabolic processes that give rise to these compounds. Ignoring the correlations between compounds due to these metabolic interactions could lead to erroneous conclusions on patterns of volatile release upon herbivory. There is potential for combining this analysis with EAG because this will indicate physiologically active compounds that should be included in the compositional analysis. We have also introduced the use of GLMs for the analysis of olfactometer data. Though GLM techniques are well established in ecology, they have not been widely used in behavior, despite their large potential. The introduction of these methods into chemical ecology could prove to be useful tools in the study of volatile-mediated interactions.

Acknowledgements We are grateful to K. Chamberlain, L. Wadhams, and M. Borges for providing help with the chemical analyses, J. Aldrich for providing synthetic standards, V. Brown for advice on the experiments, G. Piaggio for statistical advice and comments on previous versions of the manuscript, and M. Torrance and K. Plumb for help in rearing. We thank two anonymous reviewers and M. Hilker for valuable comments during the review process. This work was funded by a Lawes Trust–University of Reading award to MP. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom.

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J Chem Ecol (2007) 33:711–729
DOI 10.1007/s10886-007-9258-5

Intraspecific Variation in Aphid Resistance and Constitutive Phenolics Exhibited by the Wild Blueberry *Vaccinium darrowi*

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Received: 13 November 2006 / Accepted: 29 January 2007 /
Published online: 28 February 2007
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Abstract *Illinoia pepperi* (MacGillivray) infests cultivated highbush blueberries, *Vaccinium corymbosum* L., in the Northeastern United States. Allopatric resistance to *I. pepperi* was examined in *Vaccinium darrowi* Camp, which evolved in the absence of *I. pepperi* in the Southeastern U.S. *V. corymbosum* cv. "Elliott", was used as a susceptible control. Between population variability in *I. pepperi* resistance was assessed by measuring length of the prereproductive period, fecundity, and survivorship on 14 *V. darrowi* accessions representing 11 discrete wild populations. Length of *I. pepperi*'s prereproductive period and survivorship were not significantly affected. However, differences were detected in fecundity and the intrinsic rate of increase (r_m). Within population variability in resistance was measured by confining first instars to 24 accessions from a single wild population of *V. darrowi* (NJ88-06). Significant differences in the mean total number of aphids occurring after 20 d were only detected between 2 of the 24 *V. darrowi* accessions. A greater degree of diversity in *I. pepperi* resistance exists between populations of *V. darrowi* compared to within a population. Constitutive leaf and stem polyphenolics were identified by HPLC-MS and quantified from 14 of the *V. darrowi* accessions. The accessions varied in concentrations of five phenolic acids and seven flavonol glycosides, but a correlation was not found between individual or total phenolics and aphid performance. Overall, screening within and between populations of *V. darrowi* identified promising sources of aphid resistance, but

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phenolic acid and flavonol glycoside profiles did not predict resistance levels. The mechanism of resistance remains to be identified.

Keywords Allopatric resistance · Blueberry aphid · *Illinoia pepperi* · Metabolic profiling · *Vaccinium darrowi*

Introduction

The aphid, *Illinoia pepperi* (MacGillivary), is the dominant aphid infesting cultivated highbush blueberry, *Vaccinium corymbosum* L., in New Jersey and Michigan (Morimoto and Ramsdell, 1985; Elsner and Kriegel, 1989). The entire life cycle of *I. pepperi* is spent on a *Vaccinium* host, which may include cultivated and wild highbush blueberry, *V. corymbosum*, wild lowbush blueberry, *Vaccinium angustifolium* (Aiton), and wild velvetleaf blueberry, *Vaccinium myrtilloides* Michaux (Lockhart and Hall, 1962; Elsner and Kriegel, 1989). However, *I. pepperi* populations are highest on cultivated blueberry, and movement outside of a field to wild *Vaccinium* plants in the surrounding landscape is considered rare (Morimoto and Ramsdell, 1985).

The holocyclic life cycle of *I. pepperi* consists of overwintering eggs that hatch into wingless, parthenogenic fundatrices during the spring months (Elsner and Kriegel, 1989). Subsequent summer generations consist of alate or apterous virginoparae giving birth parthenogenetically to live young, with the highest populations occurring in June (Morimoto and Ramsdell, 1985). During the fall, sexual morphs of apterous females and alate males are produced, but Morimoto and Ramsdell (1985) collected a few alatae by using water-pan traps after mid-July.

Aphid-transmitted viruses, such as blueberry scorch virus (BlScV) and blueberry shoestring virus (BBSSV), pose economic threats to the commercial production of highbush blueberry, particularly in New Jersey, Michigan, and the Pacific Northwest (Morimoto and Ramsdell, 1985; Martin and Bristow, 1995; Hillman et al., 1996). BlScV belongs to the carlavirus group and is transmitted in a nonpersistent fashion (Hillman et al., 1996), while transmission of the sobemovirus BBSSV occurs in a persistent fashion (Ramsdell, 1979; Terhune et al., 1991). Managing the incidence of BlScV and BBSSV mainly occurs through insecticide applications to reduce aphid population densities, by rouging symptomatic plants and by planting clean root stock (Raworth, 2004). However, as BlScV has a latent period of 1–3 years before symptoms appear (Bristow et al., 2000), an infected plant could continue to serve as a source of inoculum. Therefore, simply rouging symptomatic plants does not represent an effective management strategy. Host-plant resistance can provide a multifaceted approach to vector and virus management. For example, Massonie and Maison (1986) documented reduced virus transmission by using vector-resistant peaches, *Prunus* spp., to reduce the population fitness of the green peach aphid, *Myzus persicae* (Sulzer). The use of plant resistance to limit the population growth of *I. pepperi* could aid in reducing the incidence of aphid-transmitted viruses in cultivated blueberries.

Previous studies found that high levels of insect and disease resistance were exhibited by wild *Vaccinium* spp. (Etzel and Meyer, 1986; Meyer and Ballington, 1990; Stretch et al., 2001). To identify promising sources of aphid resistance, performance parameters of *I. pepperi* were compared after being reared on several wild *Vaccinium* spp. that occur sympatrically and allopatrically with *I. pepperi* (Ranger et al., 2006). Delayed development, along with reduced fecundity and survivorship were associated with several of the *Vaccinium* spp., particularly the allopatric spp. *Vaccinium myrsinites* Lamarck, *Vaccinium*

Table 1 Between population variability in resistance of *Vaccinium darrowi* to the aphid *Illinoia pepperi*

Species and Population ID[b]	Collection Locality	Mean (±SEM)[a]		
		Prereproductive Period (TTR)[c]	Total Fecundity[d]	Survivorship (%) 42 d After Birth
V. darrowi				
NJ88-06-20	Santa Rosa Co., FL	14.7±0.9a	22.0±3.3a	66a
NJ88-15-09	Okaloosa Co., FL	14.7±1.2a	22.1±4.1a	88a
NJ88-13-22	Liberty Co., FL	14.3±0.9a	25.3±2.1ab	87.5a
NJ88-14-03	Gulf Co., FL	14.0±0.9a	29.4±3.9ab	88a
NJ88-04-64	Santa Rosa Co., FL	14.0±0.8a	30.4±4.2ab	66a
NJ88-17-13	Stone Co., MS	14.2±1.3a	34.1±5.0ab	90a
NC84-06a-37	Lake Co., FL	13.8±1.0a	35.0±4.7ab	88a
NJ88-06-01	Santa Rosa Co., FL	13.8±0.8a	36.1±4.8abc	80a
Fla 4B	Marion Co., FL	13.6±0.9a	44.9±4.9bc	100a
NJ88-12-41	Liberty Co., FL	13.3±1.1a	30.1±3.7ab	88a
NJ88-05-29	Covington Co., AL	13.3±1.0a	26.3±4.0ab	88a
NC84-06a-20	Lake Co., FL	13.3±1.0a	38.3±4.8abc	90a
NJ88-11-32	Lake Co., FL	12.5±0.7a	45.4±4.8bc	88a
NJ88-17-27	Stone Co., MS	12.2±0.6a	42.9±4.6bc	100a
V. corymbosum				
Elliott	USDA Breeding Program	11.1±0.4a	58.9±4.0c	100a

[a] Means within columns followed by the same letter are not significantly different [α±0.05; Tukey's studentized range (HSD) test]. N±9 for all accessions

[b] The first value in the population ID (e.g., NJ88-15-09) represents the year of collection, the second value represents the population number, and the third value represents the specimen number.

[c] From birth to reproductive maturity

[d] Total offspring produced over a period of 42 d after birth

hirsutum Buckley, *Vaccinium boreale* Hall and Aalders, and *Vaccinium darrowi* Camp. Wild *V. darrowi* accessions have been used to develop and improve numerous highbush cultivars (Chandler et al., 1985; Erb et al., 1990; Hancock et al., 1992), but *V. darrowi* accessions highly resistant to *I. pepperi* have yet to be identified.

As heterogeneity in resistance levels to insect herbivores occurs in wild plant populations (Fritz, 1992), we hypothesized that intraspecific variability in *I. pepperi* resistance would exist within and between populations of *V. darrowi*. Because variability in phenolics occurs within and between plant populations (Nichols-Orians et al., 1993; Hakulinen et al., 1995; Binns et al., 2002; Van Heerden et al., 2003), constitutive phenolics were also quantified to determine if such compounds could be used to predict resistance levels.

Methods and Materials

Plants and Insects A total of 37 different *V. darrowi* (2×) accessions were evaluated for within and between population variability in resistance to the blueberry aphid, *I. pepperi*, along with the standard highbush cultivar *V. corymbosum* cv. Elliott (4×) as a susceptible control. All *V. darrowi* accessions were collected from wild populations (Table 1) and are maintained in the *Vaccinium* germplasm collection at the Philip E. Marucci Center for

Blueberry and Cranberry Research and Extension, Rutgers-The State University of New Jersey, Chatsworth, NJ, USA. Collection details are described by Bruederle and Vorsa (1994). Specimens collected from different counties represent distinct populations, while those from the same county represent a single population (Table 1). The parent plants are potted in a (1:1 *v:v*) sand to peat moss mixture and maintained under greenhouse conditions. Multiple clones of each *V. darrowi* accession were established in May 2004 by excising rhizomes from individual parent plants for each accession. Clones of each *V. darrowi* accession are genetically identical. One-yr-old, *V. corymbosum* cv. Elliott plants were purchased from DeGrandchamp's blueberry farm (South Haven, MI, USA). All plants were grown in a sand: peat moss (1:1 *v:v*) mixture and maintained in a greenhouse for approximately 6 mo before their use in experiments. Osmocote 14–14–14 (N:P:K) (Scotts-Sierra Horticultural Products, Marysville, OH, USA) was used as a controlled release fertilizer.

A colony of *I. pepperi* was established in June 2004 by collecting several apterous *I. pepperi* virginoparae from a commercial blueberry planting in Atlantic Co., NJ, USA. As the overall objective was to identify allopatric sources of resistance against *I. pepperi*, multiple *I. pepperi* clones were used to establish and maintain the colony. Apterous *I. pepperi* were occasionally added to the colony to ensure the presence of multiple genotypes. Aphids were reared on excised shoots of *V. corymbosum* cv. Elliott and maintained in a laboratory under fluorescent lights with a photoperiod of 16:8 (L:D).

Between Population Variability in Aphid Resistance Heterogeneity in resistance of *V. darrowi* to *I. pepperi* was measured by using 14 *V. darrowi* accessions representing 11 discrete populations (Table 1), along with *V. corymbosum* cv. Elliott as a susceptible control. Three adult apterous *I. pepperi* were collected from the laboratory-reared colony and transferred to dialysis-tube cages (4-cm diameter, 13-cm long; Ward's National Science, Rochester, NY, USA), containing approximately 6–7 cm of an actively growing shoot, including the apical meristem, from a particular *Vaccinium* accession. Foam plugs were used to seal both ends of the cage, with the bottom plug being cut along the radius to allow for a single stem to enter and be sealed within the dialysis tube. Cages contained leaf and stem tissue and were positioned on the plants 24 hr before their use. The experiment was performed twice, and a total of nine replications were used for each accession.

Plants were arranged in a completely randomized design on a single greenhouse bench. Cages were inspected daily until nymphiposition began, at which point all individuals were removed except for two first instars. Nymphs were allowed to develop until one reached reproductive maturity, after which the second nymph was removed. Fecundity was subsequently measured every 2 d until 42 d after birth. This procedure allowed for calculating the length of time from birth to reproductive maturity, total offspring production, and survivorship over a 42-d period.

The intrinsic rate of natural increase (r_m) was estimated using the equation of Wyatt and White (1977): $r_m = 0.74(\ln M_d)/\mathrm{TTR}$, where 0.74 is a correcting constant, M_d is the number of young produced in a reproductive period equal to *TTR*, and *TTR* is the time from birth to reproduction (Wyatt and White, 1977). Doubling time ($T_d = \ln 2/r_m$), net reproductive rate ($R_o = \sum l_x m_x$), and generation time ($T = \ln R_o/r_m$) were also calculated, whereby l_x=age-specific survivorship and m_x=age-specific fecundity (Andrewartha and Birch, 1954; DeLoach, 1974). As all aphids were monitored from birth, the age specificity of each aphid was known.

Within Population Variability in Aphid Resistance Variation in aphid resistance within a population of *V. darrowi* was measured with 24 accessions from a single *V. darrowi*

population (NJ88-06) collected in Santa Rosa Co., FL, USA, namely, NJ88-06-01; -02; -03; -04; -05; -07; -08; -11; -12; -14; -17; -22; -23; -25; -27; -38; -39; -41; -42; -43; -44; -46; -49; and -51. *Vaccinium corymbosum* cv. Elliott was also used as a susceptible highbush control. Plants were arranged in a completely randomized design on a single greenhouse bench.

Three adult apterous *I. pepperi* were collected from the laboratory-reared colony and transferred to the previously described dialysis-tube cages. Inspections were made until nymphiposition occurred in each cage, at which point, the adults and all but two first instars were removed. As the instars neared reproductive maturity, one nymph was removed, leaving behind a single, robust *I. pepperi* nymph in each cage. Twenty days after birth of the first instars, the total numbers of aphids in each cage were recorded on the *V. darrowi* accessions and *V. corymbosum* cv. Elliott. The experiment was performed twice, and a total of eight replications were used for each accession. Due to the large number of NJ88-06 accessions and replicates of each, it was not feasible to document developmental rates and fecundity as in the between population experiment.

Extraction, Quantification, and Identification of Phenolics Actively growing shoots from each *V. darrowi* accession representing 11 discrete populations, along with *V. corymbosum*, were harvested on three separate occasions during May–June 2005. Excised shoots were placed in paper bags and set in a drying oven set to 60±5°C for 24 hr. After drying, leaves were separated from stems, and each was ground into a homogenous powder. Based on Witzell et al. (2003), 30 mg of leaf or stem tissue were placed in an Eppendorf tube with 1,000 μl of chilled methanol (MeOH). Samples were placed on a shaker (Labnet Shaker 20, Labnet International Inc., Edison, NJ, USA) at 1,000 rpm for 2 min, and then centrifuged at 12,000 rpm for 5 min (Eppendorf centrifuge 5417C, Eppendorf, Hamburg, Germany). The supernatant was collected, and the tissues were re-extracted as described above with 500 μl of solvent. Both supernatants were pooled, filtered, and concentrated to dryness with a Speed Vac concentrator (Thermo Model SPD 2010-220; Milford, MA, USA). Residues were redissolved in 1 ml of MeOH.

Selected phenolic acids and flavonols in leaf and stem extracts were identified by high performance liquid chromatography coupled with mass spectrometry (HPLC-MS). The HPLC system (Shimadzu Co., 10VP Series, Columbia, MD, USA) employed a C_{18} MCM column (4.6×150 mm; 5 μm; MC Medical, Inc, Tokyo, Japan). Twenty microliters were injected into the column, and a gradient elution was used for separations. Solvent A consisted of 10% MeOH in H_2O adjusted to pH 3.5 with formic acid. Solvent B consisted of 20% H_2O (pH 3.5), 20% MeOH, and 60% acetonitrile. At a flow rate of 0.3 ml min^{-1}, the following gradient was used: 0 min, 100% A; 10 min 20% A; 20 min, 40% A; 40 min, 0% A; held at 0% A for 15 min. Five minutes of equilibration at 100% A was performed before and after each injection. Effluent from the column was introduced into a single-quadrupole mass spectrometer (API 150 MCA, PE Sciex, Concord, Ontario, CA) equipped with electrospray ionization (ESI). The following parameters were used: capillary voltage at 5,500 V, nebulizer gas (N_2) at 9 (arbitrary units), curtain gas (N_2) at 10 (arbitrary units), collision gas (N_2) at 10 (arbitrary units), focusing potential at 300 V, entrance potential at 10 V, declustering potential at 75, drying gas (N_2) heated to 320°C and introduced at a flow rate of 8,000 cm^{-3} min/l. Data were acquired in both positive and negative ionization mode, with full scan acquisition being performed from *m/z* 50–1,200. Data were processed by Analyst software 1.4.1. (Applied Biosystems, Applera Co., Foster City, CA, USA).

Retention times, spectral patterns, and Electrospray ionization mass spectrometry (ESI-MS) fragmentations patterns of 5-caffeoylquinic acid and the flavonol glycosides were compared with

Table 2 Population fitness of *Illinoia pepperi* confined to wild accessions of *Vaccinium darrowi* representing distinct populations, along with *V. corymbosum* cv. Elliott

Species and Population ID	Intrinsic Rate of Increase (r_m)[a,b]	95% CI[b]	Net Reproductive Rate (R_o)[c]	Generation Time (T)[d]	Doubling Time (T_d)[e]
V. darrowi					
NJ88-06-20	0.132a	0.098–0.163	18.3	20.4	3.8
NJ88-15-09	0.140ab	0.103–0.178	21.5	21.9	4.9
NJ88-13-22	0.145a	0.130–0.162	25.1	22.2	4.8
NJ88-05-29	0.150ab	0.129–0.175	20.9	20.3	4.6
NJ88-14-03	0.150ab	0.128–0.173	28.8	22.5	4.6
NJ88-12-41	0.160ab	0.134–0.187	29.9	21.3	4.3
NC84-06a-37	0.160ab	10.130–0.187	32.5	21.8	4.3
NJ88-17-13	0.161ab	0.134–0.191	36.2	22.0	4.2
NJ88-04-64	0.163ab	0.141–0.185	28.7	20.7	4.3
NJ88-06-01	0.173abc	0.146–0.201	37.7	20.0	3.8
NC84-06a-20	0.176abc	0.145–0.207	39.7	20.4	3.8
Fla 4B	0.188bc	0.172–0.204	46.8	20.5	3.7
NJ88-17-27	0.191bc	0.165–0.232	41.6	19.6	3.6
NJ88-11-32	0.192bc	0.165–0.220	41.7	19.4	3.6
V. corymbosum					
Elliott	0.216c	0.198–0.232	59.9	18.9	3.2

[a] r_m=0.74(ln M_d)/TTR, where M_d is the number of young produced in a reproductive period equal to TTR, and *TTR* is the time from birth to reproductive maturity. Bootstrap estimate of r_m. N=9 for all accessions.

[b] Nonoverlapping 95% CI corresponds to the rejection of no treatment effect ($\alpha \pm 0.05$).

[c] $R_o = \sum l_x m_x$

[d] $T = \ln R_o / r_m$

[e] $T_d = \ln(2)/r_m$

authentic standards. Authentic standards of 5-caffeoylquinic acid, and quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, and quercetin-3-rutinoside were purchased from Indofine Chemical Company, Inc. (Somerville, NJ, USA). The remaining phenolic acids were identified by interpreting ESI-MS fragmentation patterns and by comparing with published data (Clifford et al., 2003; Cho et al., 2004; Vvedenskaya et al., 2004).

The selected phenolics were quantified with an HPLC (Waters, Milford, MA, USA) equipped with a Waters 996 photodiode array detector (PDA). All phenolics were quantified by using the external standard method, with quantification being based on peak area. Quantification of the chlorogenic acid derivatives was based on a standard curve prepared with 5-caffeoylquinic acid. Quercetin derivatives were quantified by using the corresponding authentic standard for each compound, except quercetin-3-oxalylpentoside and quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside were based on a standard curve prepared with quercetin-3-rutinoside. For both the standards and extracts, 20 μl were injected and separated by using the aforementioned column and solvent system, but at a flow rate of 1 ml/min. Scanning by the PDA occurred at 320 nm, while data acquisition and processing were performed by Waters Empower™ Chromatography Software.

Statistical Analyses Aphid performance parameters and phenolic concentrations were analyzed with one-way analysis of variance (ANOVA) (PROC GLM; SAS Institute, 1996).

Table 3 Retention times, λ_{max}, *m/z* values, molecular ions, and fragmentation patterns of polyphenolics isolated from *Vaccinium darrowi* and *V. corymbosum* cv. Elliott

RT (min)	λ_{max} (nm)	$[M-H]^-$ and Fragmentation in Electrospray-MS	$[M+H]^+$ and Fragmentation in Electrospray-MS	Structure
17.38	216, 243, 324	353, 191, 178.9, 127, 111	355, 193, 163, 127	3-Caffeoylquinic acid[a]
19.65	216, 243, 324	353, 191, 178.9, 127, 111	355, 193, 163, 127	5-Caffeoylquinic acid[a,b]
20.49	220, 227, 312	337, 190.7, 161, 119	ND	3-*O*-*p*-Coumaroylquinic acid[a]
23.26	210, 227, 312	337, 191, 161, 127	ND	5-*O*-*p*-Coumaroylquinic acid[a]
24.62	218, 295, 323	433, 338.6, 322.8, 161	435, 340.2, 324.1, 162.1	Unidentified
25.46	203, 255, 354	609, 301	611, 303	Quercetin-3-rutinoside[b,c]
26.09	203, 255, 352	463, 301	465, 303	Quercetin-3-galactoside[b,c,d]
26.29	203, 255, 352	463, 301	465, 303	Quercitin-3-glucoside[b,c,d]
26.80	220, 288, 335	594.8, 433, 301	596, 435, 303	Quercetin-3-glucosylpentoside[b,c]
28.51	223, 312	505, 433, 300	507, 435, 303	Quercetin-3-oxalylpentoside[b,c]
28.88	253, 344	447, 301	449, 303	Quercetin-3-rhamnoside[b,c,d]
31.57	205, 257, 353	609, 463, 301	611, 465, 303	Quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside[d]

[a] In conjunction with Clifford et al. (2003)

[b] Based on comparisons with authentic standards

[c] In conjunction with Cho et al. (2004)

[d] In conjunction with Vvedenskaya et al. (2004)

To help stabilize the variance, data were square root transformed before analysis, but untransformed data are presented. Means were separated by using Tukey's studentized range [honestly significant difference (HSD)] test at α=0.05. Pearson's Correlation coefficient was calculated to detect a correlation between aphid performance parameters (i.e., length of prereproductive period or cumulative fecundity) and individual or total phenolics (PROC CORR; α=0.05; SAS Institute, 1996). The percentage of aphids surviving on each *Vaccinium* spp. 42 d after birth was compared by using a R×C

contingency table with PROC FREQ procedure (SAS Institute, 1996), and Fisher's exact test (two-tail) was used to test for significant differences between observed and expected frequencies (α=0.05).

The bootstrap technique was used to estimate 95% confidence intervals (CI) for r_m values associated with each *Vaccinium* spp. (Meyer et al., 1986; Petitt et al., 1994; R Project for Statistical Computing, 2005). Nonoverlapping 95% CI corresponds to the rejection of no treatment effect hypothesis at α=0.05 (Maia et al., 2000). As T_d and T are functions of r_m, and R_0 represents the population's response, statistical analyses were only performed on r_m; however, values for each are provided for comparative purposes.

Results

Between Population Variability in Aphid Resistance Length of the prereproductive period of *I. pepperi* (from birth to reproductive maturity) was not significantly affected when reared on the 14 *V. darrowi* accessions representing 11 populations or *V. corymbosum* cv. Elliott (Table 1; P>0.05). Among the *V. darrowi* accessions, length of the prereproductive period ranged from a high of 14.7 d on NJ88-06-20 and NJ88-15-09 to a low of 12.2 d on NJ88-17-27 (Table 1). The shortest time required until reproductive maturity (11.1 d) occurred on *V. corymbosum* (Table 1).

Cumulative mean fecundity of *I. pepperi* up to 42 d after birth was affected by the *Vaccinium* host (Table 1; F=5.15; df=27, 106; P<0.001). On the *V. darrowi* accessions, cumulative fecundity ranged from 22.0 nymphs on NJ88-06-20 to 45.4 nymphs on NJ88-11-32. The two lowest fecundities were associated with *V. darrowi* accessions NJ88-06-20 and NJ88-15-09, which differed significantly from NJ88-11-32, NJ88-17-27, Fla 4B, and *V. corymbosum*. The highest cumulative nymphal production of 58.9 nymphs was observed with *V. corymbosum*, which was significantly different from all of the *V. darrowi* accessions except NJ88-17-27, NJ88-11-32, NC84-06a-20, Fla 4B, and NJ88-06-01 (Table 1). Percent survivorship, 42 d after birth, ranged from 66% on NJ88-06-20 and NJ88-04-64 to 100% on *V. darrowi* Fla 4B, NJ88-17-27, and *V. corymbosum* (Table 1), but significant differences were not detected.

By using the length of the prereproductive period and the number of offspring produced over an equivalent amount of time, the intrinsic rate of increase (r_m), net reproductive rate (R_0), generation time (T), and doubling time (T_d) were calculated for *I. pepperi* when reared on the *V. darrowi* accessions and *V. corymbosum* (Table 2). Longer prereproductive periods and reduced offspring production resulted in the r_m values of *V. darrowi* NJ88-06-20 (r_m=0.132) and NJ88-13-22 (r_m=0.145) being significantly lower than those associated with *V. darrowi* Fla 4B (r_m=0.188), NJ88-11-32 (r_m=0.192), and *V. corymbosum* (r_m=0.216) (Table 2).

Considerable variability in *I. pepperi* net reproductive rates (R_0) was observed across the *V. darrowi* accessions, which represented populations across the species geographic range (Table 2). Among the *V. darrowi* accessions, the lowest net reproductive rate of R_0=18.3 occurred on *V. darrowi* NJ88-06-20, while the highest of R_0=46.8 occurred on *V. darrowi* Fla 4B (Table 2). Generation times (T) among the *V. darrowi* accessions ranged from a high of 22.5 to a low of 19.4 on *V. darrowi* NJ88-14-03 and *V. darrowi* NJ88-11-32, respectively. Doubling time (T_d) ranged from 4.9 for *V. darrowi* NJ88-15-09 to 3.6 for both *V. darrowi* NJ88-11-32 and NJ88-17-27 (Table 2). Population fitness of *I. pepperi* was best on *V. corymbosum*, which exhibited the highest R_0 (59.9) and the lowest T (18.9) and T_d (3.2) (Table 2).

Within Population Variability in Aphid Resistance As *I. pepperi* exhibited comparatively poor performance and population fitness on *V. darrowi* accession NJ88-06-20 compared with representatives from other *V. darrowi* populations (Tables 1 and 2), variability in aphid resistance was compared among 24 accessions collected from a single *V. darrowi* population (NJ88-06; Santa Rosa Co., FL). The mean total number of aphids (occurring 20 d after the confinement of a newly born first instar) was higher on *V. corymbosum* cv. Elliott (17.6±2.1) compared to all of the *V. darrowi* accessions (F=4.24; df=24, 175; P<0.001; data not shown). The fewest individuals were recorded from *V. darrowi* NJ88-06-20 (1.1±0.3 aphids). However, significant differences were only detected between 2 of the 24 *V. darrowi* accessions, namely, NJ88-06-20 and (1.1±0.3 aphids) and NJ88-06-51 (6.6± 1.5 aphids).

Identification and Quantification of Phenolics ESI-MS performed under both positive $[M+H]^+$ and negative $[M-H]^-$ modes allowed molecular masses and fragmentation patterns to be identified for several chlorogenic acid and quercetin derivatives extracted from *V. darrowi* and *V. corymbosum* cv. Elliott leaves and stems (Table 3). All of the identified compounds exhibited characteristic ESI-MS profiles and matched well with published data (Clifford et al., 2003; Cho et al., 2004; Vvedenskaya et al., 2004).

Four chlorogenic acid derivatives were identified, namely, 3- and 5-caffeoylquinic acid, and 3- and 5-*O*-*p*-coumaroylquinic acid (Table 3). 5-Caffeoylquinic acid was identified by comparing retention times and ESI-MS fragmentation patterns with an authentic standard, while the remaining chlorogenic acid derivatives were identified by interpreting their ESI-MS fragmentation patterns, parent and daughter ions, along with comparisons with published data (Clifford et al., 2003). A fifth chlorogenic acid derivative (RT 24.62 min; Table 3) was not definitively identified, but the presence of $[M-H]^-$ ions at *m/z* 338.6 and *m/z* 161 indicated a *p*-coumaric acid unit. For all of the chlorogenic acid derivatives, an intermediate ion was present that corresponded to the loss of caffeic acid or *p*-coumaric acid units, as indicated by the $[M-H]^-$ and $[M-H]^+$ fragmentation patterns (Table 3).

Seven flavonol glycosides were identified from leaves and stems of *V. darrowi* and *V. corymbosum* (Table 3), namely, quercetin-3-rutinoside, quercetin-3-glucoside, quercetin-3-glucosylpentoside, quercetin-3-oxalylpentoside, quercetin-3-rhamnoside, and quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside. For each of the flavonol glycosides, the aglycone component was indicated by the prominent ion produced following the loss of the sugar moieties from the pseudo-molecular ion ($[M-H]^-$; Table 3). By maintaining a low source temperature and a high declustering potential in ESI-MS conditions, peak sizes of the daughter ions were intensified. Consequently, the relative intensities of the molecular ion peaks were lower compared to the daughter ion peak, which allowed for the identification of each of the sugar moieties (Table 3; Cho et al., 2004; Vvedenskaya et al., 2004). Identifications of all the flavonol glycosides were based on comparing ESI-MS fragmentation patterns and retention times with commercial standards (Table 3), except for quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside, which was based on interpreting ESI-MS fragmentation and comparisons with published data.

The five chlorogenic acid derivatives and seven quercetin derivatives were quantified from leaf and stem tissues of *V. darrowi* and *V. corymbosum* (Tables 4 and 5). Comparisons of individual chlorogenic acid and quercetin derivatives revealed considerable variability between representatives of the distinct *V. darrowi* populations, except for quercetin-3-rutinoside extracted from stem tissues. For example, in leaves of *V. darrowi* NC84-6a-20 and NJ88-06-01, 5-caffeoylquinic acid ranged from 3,001 to 257 μg/g of leaf tissue, respectively (Table 4). Overall, 5-caffeoylquinic acid was among the more abundant leaf

Table 4 Chlorogenic acid and quercetin derivatives extracted from leaves of wild populations of *Vaccinium darrowi* and *V. corymbosum* cv. Elliott

Species and Population ID	Mean (±SEM) Concentration (μg of 5-Caffeoylquinic Acid Equivalent/g of Leaf Tissue)[a]					Mean (±SEM) Concentration (μg of Quercetin Equivalent/g of Leaf Tissue)[b]						
	3-Caffeoylquinic Acid	5-Caffeoylquinic Acid	3-*O*-*p*-Coumaroyl-quinic Acid	5-*O*-*p*-Coumaroyl-quinic Acid	Unidentified Chlorogenic Acid Derivative	Quercetin-3-rutinoside	Quercetin-3-galactoside	Quercetin-3-glucoside	Quercetin-3-glucosyl pentoside	Quercetin-3-oxalyl-pentoside	Quercetin-3-rhamnoside	Quercetin-3-*O*- (6-*p*-coumaroyl)-β-galactoside
V. darrowi												
NJ88-04-64	1403.9± 0.4a	1019.2± 134.2cd	40.4± 11.3e	150.3± 10.6abc	981.1± 19.0abc	122.4± 10.4abcd	19.1± 3.3abc	79.9± 20.7bc	1123.7± 29.8ab	0.0± 0.0e	447.7± 306.7a	511.0± 30.2bcd
NJ88-05-29	229.9± 33.7bcd	355.8± 107.9ef	213.2± 29.1bc	66.9± 9.7cdef	0.0± 0.0g	49.1± 3.6de	10.9± 2.6bcd	47.8± 3.6cd	1155.0± 164.2ab	44.2± 7.5e	157.4± 14.1ab	574.6± 34.5abc
NJ88-06-01	31.9± 1.4e	257.1± 23.5f	107.3± 9.9cde	203.2± 14.9a	365.5± 26.4de	176.9± 67.4abc	2.3± 0.7e	121.0± 9.6ab	54.0± 13.3de	755.6± 120.0bcd	89.9± 1.8ab	397.2± 76.4bcd
NJ88-06-20	99.2± 20.4cde	644.4± 3.2de	151.4± 26.4bcd	27.2± 5.8f	271.9± 24.0e	155.2± 31.6abcd	19.8± 5.4abc	33.6± 7.4de	45.2± 4.1de	676.5± 62.0d	124.3± 0.3ab	330.0± 14.4cde
NJ88-11-32	59.9± 1.4de	382.5± 35.5ef	157.5± 27.8bcd	43.4± 8.4ef	453.9± 14.3de	90.0± 15.6cde	22.0± 1.4ab	13.6± 2.4ef	1106.3± 139.0ab	782.2± 169.3bcd	120.9± 12.8ab	638.1± 24.1ab
NJ88-12-41	360.0± 87.2b	637.2± 24.3de	48.0± 5.6de	157.3± 17.7ab	382.6± 41.9de	125.0± 17.4abcd	17.5± 0.4abc	0.0± 0.0g	0.0± 0.0f	1303.9± 49.9a	123.4± 16.1ab	655.6± 39.9ab
NJ88-13-22	293.8± 97.1bc	624.2± 125.2de	301.3± 54.8ab	43.0± 10.5ef	1083.9± 102.9ab	97.7± 4.1cde	8.6± 0.4cde	49.4± 20.4cd	42.0± 2.1def	1193.2± 41.6abc	60.6± 2.2b	283.6± 51.9de

NJ88-14-03	363.9± 31.9b	935.7± 57.2d	63.2± 16.3de	64.1± 16.3def	466.9± 29.8d	246.6± 32.3ab	11.1± 1.51bcd	185.6± 20.9a	531.2± 4.8c	22.3± 8.3e	64.6± 3.9b	165.0± 9.2e
NJ88-15-09	68.0± 6.5de	549.2± 23.4def	244.1± 58.0bc	182.6± 46.5ab	370.5± 57.2de	105.6± 21.7bcde	5.7± 0.9de	60.8± 2.7cd	751.9± 119.0bc	744.5± 84.5cd	72.3± 4.8b	424.5± 20.5bcd
NJ88-17-13	18.3± 6.5e	407.7± 0.9ef	94.7± 1.7cde	22.2± 6.6f	785.2± 114.9c	247.5± 15.1ab	14.4± 3.3bcd	44.4± 1.3cd	33.8± 6.6ef	826.4± 113.9abcd	83.9± 9.0ab	580.8± 45.5abc
NJ88-17-27	348.7± 14.7b	959.0± 218.9d	144.2± 35.2bcde	99.5± 8.0bcde	1160.3± 84.2a	132.1± 16.7abcd	9.0± 2.0cde	0.0± 0.0g	1243.7± 38.5a	1230.1± 118.0ab	38.5± 4.1b	532.9± 138.3bcd
NC84-6a-20	348.3± 41.6b	3001.0± 176.8a	459.2± 61.1a	124.9± 22.3abcd	1036.4± 23.8abc	226.7± 42.7abc	12.8± 1.7bcd	0.0± 0.0g	713.1± 49.0c	50.9± 4.9e	138.0± 24.9ab	561.5± 80.4bcd
NC84-6a-37	366.6± 53.6b	1585.7± 62.4bc	132.4± 20.7cde	36.9± 1.9ef	799.7± 6.0bc	278.6± 57.4a	13.2± 1.2bcd	57.1± 9.5cd	160.1± 6.6d	986.6± 84.3abcd	138.2± 6.9ab	857.5± 37.8a
Fla 4B	335.8± 103.6b	399.2± 22.0ef	99.7± 16.9cde	45.0± 11.5ef	75.0± 1.6f	23.6± 3.2e	28.4± 3.0a	2.8± 2.8fg	0.0± 0.0f	955.7± 128.0abcd	47.4± 8.8b	334.5± 20.0cde
V. corymbosum												
Elliott	183.0± 7.5bcd	1852.3± 7.5b	158.7± 12.6bcd	23.7± 8.4f	11.7± 0.8g	115.3± 12.3bcd	2.7± 0.1e	85.1± 3.1bc	10.6± 1.2ef	29.7± 7.2e	38.5± 4.3b	453.8± 1.2bcd
F and *P* value	29.56; <0.001	43.37; <0.001	13.07; <0.001	17.19; <0.001	124.22; <0.001	8.71; <0.001	12.72; <0.001	53.89; <0.001	113.24; <0.001	76.21; <0.001	2.96; =0.0062	11.01; <0.001

[a] Means within columns followed by the same letter are not significantly different [α=0.05; Tukey's studentized range (HSD) test; *df*=14, 30]. *N*=3 for all accessions. Chlorogenic acid derivatives are expressed as μg of 5-caffeoylquinic acid equivalent per g dry weight.

[b] Means within columns followed by the same letter are not significantly different (α=0.05; Tukey's studentized range [HSD] test; *df*=14, 30). *N*=3 for all accessions. Quantity of each quercetin derivative based on corresponding standards, except quercetin-3-oxalylpentoside and quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside were based on quercetin-3-rutinoside.

Table 5 Chlorogenic acid and quercetin derivatives extracted from stems of wild populations of *Vaccinium darrowi* and *V. corymbosum* cv. Elliott

Species and Population ID	Mean (±SEM) Concentration (μg of 5-Caffeoylquinic acid Equivalent/g of Stem Tissue)[a]					Mean (±SEM) Concentration (μg of Quercetin Equivalent/g of Stem Tissue)[b]						
	3-Caffeoylquinic Acid	5-Caffeoylquinic Acid	3-*O*-*p*-Coumaroyl-quinic Acid	5-*O*-*p*-Coumaroyl-quinic Acid	Unidentified Chlorogenic Acid Derivative	Quercetin-3-rutinoside	Quercetin-3-galactoside	Quercetin-3-glucoside	Quercetin-3-pentoside	Quercetin-3-oxalyl-pentoside	Quercetin-3-rhamnoside	Quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside
V. darrowi												
NJ88-04-64	1.1± 1.1ef	15.3± 15.3b	51.8± 13.1ab	18.9± 2.1b	297.3± 15.0a	26.0± 2.3a	2.1± 0.6ab	4.5± 4.5c	0.0± 0.0c	322.7± 82.6a	65.9± 7.0a	135.1± 35.7ab
NJ88-05-29	0.1± 0.1f	82.9± 8.5ab	28.8± 6.7ab	9.4± 4.3b	108.1± 9.5a	29.2± 5.6a	1.8± 0.0ab	21.5± 5.6bc	143.4± 25.8b	1.3± 1.3d	26.8± 4.2b	63.4± 6.7bcd
NJ88-06-01	28.4± 1.8bc	193.9± 54.7a	61.2± 19.3ab	10.2± 2.7b	98.3± 26.8a	21.0± 4.2a	3.2± 1.1ab	1.3± 1.3c	0.0± 0.0c	169.7± 6.7abcd	18.5± 4.5bc	79.1± 2.2abcd
NJ88-06-20	19.3± 1.6bcd	133.6± 27.3ab	26.5± 4.2ab	9.1± 1.1b	316.2± 25.3a	20.7± 6.2a	3.4± 0.5ab	1.1± 1.1c	118.5± 18.7b	221.5± 22.5abc	12.6± 1.3bc	54.0± 6.5cd
NJ88-11-32	17.4± 5.8bcde	72.1± 4.9ab	31.5± 11.0ab	26.7± 5.0ab	66.5± 1.9a	14.4± 4.2a	3.5± 0.3ab	1.9± 1.2c	267.5± 27.4a	223.8± 4.4abc	4.2± 0.5c	65.4± 6.2bcd
NJ88-12-41	0.2± 0.2f	120.1± 52.2ab	39.3± 10.7ab	13.6± 2.4b	95.7± 11.1a	14.3± 1.8a	3.3± 1.0ab	16.7± 5.9bc	4.5± 4.5c	108.3± 6.8cd	12.2± 2.1bc	83.2± 11.1abcd
NJ88-13-22	5.8± 5.1def	89.8± 36.6ab	51.4± 11.9ab	11.8± 3.2b	81.1± 1.0a	32.5±± 2.1a	3.7± 1.2ab	0.0± 0.0c	185.0± 18.2ab	123.1± 11.0bcd	4.1± 1.1c	113.1± 19.1abc

NJ88-14-03	2.3± 2.3ef	66.2± 5.0ab	28.6± 10.0ab	9.4± 5.6b	99.7± 20.2a	38.7± 5.8a	1.1± 0.2b	2.8±± 2.8c	9.6± 9.6c	169.0± 27.3abcd	18.2± 8.1bc	86.3± 7.7abcd
NJ88-15-09	0.0± 0.0f	71.3± 27.5ab	6.0± 3.1ab	8.4± 0.7b	91.2±± 22.8a	15.9± 8.0a	5.0± 2.3ab	1.3± 1.3c	158.3± 32.0b	90.4± 13.7cd	10.6± 0.5bc	87.7± 11.5abcd
NJ88-17-13	53.7± 13.3ab	89.6± 26.0ab	75.9± 24.0a	45.3± 12.2a	175.8± 5.3a	44.1± 25.3a	7.0± 1.5a	9.1± 4.8bc	3.2± 3.2c	284.9± 6.5ab	12.3± 4.9bc	117.5± 1.3abc
NJ88-17-27	8.1± 4.7cdef	204.8± 44.9a	42.0± 13.9ab	26.5± 9.4ab	164.2± 59.7a	16.6± 8.9a	3.2± 0.9ab	31.7± 10.2b	0.0± 0.0c	218.9± 80.7abc	8.0± 2.4bc	72.5± 15.5abcd
NC84-6a-20	16.8± 5.1bcde	154.4± 63.3ab	22.9± 12.4ab	24.9± 4.6b	96.8± 38.5a	22.3± 9.0a	3.7± 1.2ab	0.5± 0.5c	173.7± 34.8b	2.8± 2.8d	14.4± 4.7bc	143.0± 15.9a
NC84-6a-37	1.8± 1.4def	72.0± 13.5ab	0.0± 0.0b	16.4± 3.0b	147.9± 21.4a	20.4± 16.4a	5.3± 2.3ab	0.0± 0.0c	0.9± 0.9c	2.0± 2.0d	3.0± 0.8c	72.6± 25.4abcd
Fla 4B	81.7± 3.4a	111.0± 27.8ab	38.8± 20.4ab	3.1± 1.0b	12.9± 1.1a	9.0± 3.6a	5.5± 0.3ab	69.7± 7.8a	0.0± 0.0c	162.0± 29.3abcd	1.2± 0.5c	32.3± 5.1d
V. corymbosum												
Elliott	0.9± 0.6ef	34.7± 5.2ab	12.8± 9.5ab	5.2± 3.1b	0.0± 0.0a	25.4± 7.3a	1.3± 0.3ab	15.0± 3.9bc	153.5± 0.8b	6.2± 1.0d	5.5± 0.3c	74.5± 2.7abcd
F and *P* value	17.79; <0.001	3.24; = 0.0034	4.22; = <0.001	4.87; <0.001	5.24; <0.001	1.02; = 0.46	2.25; = 0.03	11.24; <0.001	51.26; <0.001	28.25; <0.001	13.50; <0.001	4.62; <0.001

[a] Means within columns followed by the same letter are not significantly different [α=0.05; Tukey's studentized range (HSD) test; *df*=14, 30]. *N*=3 for all accessions. Chlorogenic acid derivatives are expressed as micrograms of 5-caffeoylquinic acid equivalent per g dry weight.

[b] Means within columns followed by the same letter are not significantly different [α=0.05; Tukey's studentized range (HSD) test; *df*=14, 30]. *N*=3 for all accessions. Quantity of each quercetin derivative based on corresponding standards, except quercetin-3-oxalylpentoside and quercetin-3-*O*-(6-*p*-coumaroyl)-β-galactoside were based on quercetin-3-rutinoside

and stem phenolic acid. For example, 5-caffeoylquinic acid represented 83% of the chlorogenic acid derivatives extracted from leaves of *V. corymbosum*.

In addition to differences between populations of *V. darrowi*, variability was also detected among *V. darrowi* accessions from the same population. For instance, of the 12 quantified leaf phenolics, significant differences were detected for four compounds between NJ88-06-01 and NJ88-06-20, nine compounds between NJ88-17-13 and NJ88-17-27, and eight compounds between NC84-6a-20 and NC84-6a-37 (Table 4). Regarding stem phenolics, significant differences were detected for two compounds between NJ88-06-01 and NJ88-06-20, five compounds between NJ88-17-13 and NJ88-17-27, and four compounds between NC84-6a-20 and NC84-6a-37 (Table 5). In general, phenolic acid and flavonol glycoside concentrations were lower in stems compared to leaves (compare Tables 4–5).

To determine if concentrations of a particular compound predict levels of aphid resistance exhibited by *V. darrowi*, Pearson's correlation coefficients were calculated for each individual leaf and stem phenolic and aphid performance (i.e., length of prereproductive development or cumulative fecundity). A significant correlation was not detected for any of the 48 comparisons ($P>0.05$). Similarly, totals of the 12 phenolics quantified in this study did not correspond with resistance levels of the *V. darrowi* accessions ($P>0.05$).

Discussion

Allopatric resistance is genetically based resistance to insect pests that occurs between plants and insects that did not coevolve with each other (Harris, 1975; Kennedy and Barbour, 1992; Smith, 2005). Such is the case with *I. pepperi* and *V. darrowi*. *Illinoia pepperi* occurs in the Northeastern and upper Midwestern U.S. (Elsner and Kriegel, 1989; Blackman and Eastop, 2000), while *V. darrowi* occurs in the Southeastern U.S., occupying Florida, Georgia, and westward to eastern Texas (Lyrene and Sherman, 1980; Lyrene, 1997). Harris (1975) argued that allopatric resistance has been the most successful source of host plant resistance to insects for agricultural crops. Furthermore, due to the lack of gene-for-gene interaction between the pest and host plant, allopatric resistance is often polygenic and typically more stable (Harris, 1975; Smith, 2005). Allopatric resistance could be the result of genetic drift, immigration, or genes conferring resistance to insect herbivores found in the plant's native habitat (Kennedy and Barbour, 1992).

Resistance to a variety of abiotic and biotic stresses and the ability to be successfully crossed with *V. corymbosum* (Ortiz et al., 1999; Qu and Vorsa, 1999), has made *V. darrowi* of particular interest for improving the resistance properties of cultivated highbush blueberry, *V. corymbosum* (Chandler et al., 1985; Erb et al., 1990; Hancock et al., 1992). Fla 4B, reported as a *V. darrowi*, has been extensively used to improve highbush blueberry (Draper, 1995; Qu and Hancock, 1997). However, Fla 4B is not rhizomatous, which is characteristic of *V. darrowi* (Vander Kloet, 1977). Instead, Fla 4B appears to be the result of introgression between *V. darrowi* and *V. corymbosum*. Fla 4B was not identified as a promising source of aphid resistance traits in this study or others (Ranger et al., 2006).

V. darrowi is not unique compared to other *Vaccinium* spp. in having considerable variation for phenotypic and horticultural traits within and between wild populations (Camp, 1945; Lyrene and Sherman, 1980; Lyrene, 1997). The current study further supports this phenomenon as reflected in *V. darrowi* intra- and interpopulational variability for aphid resistance and phenolics. Such variation is likely attributed to genetic drift in

individual populations, introgression with sympatric *Vaccinium* spp., and/or by environmental differences between plant individuals (Vander Kloet, 1977; Lyrene and Sherman, 1980; Karban, 1992). By characterizing the intraspecific variability in *I. pepperi* resistance exhibited within and between populations of *V. darrowi*, discrete populations and individual accessions with promising levels of aphid resistance were identified. Identifying such individuals is useful for developing and improving resistance levels in future cultivars. While considering the variability in horticultural traits exhibited by wild *Vaccinium* spp., Lyrene and Sherman (1980) noted that selecting the best accession for a particular trait within a species is almost as important as selecting the most appropriate species.

Offspring production was the only biological parameter of *I. pepperi* that varied among populations of *V. darrowi*, which may be attributed to a phloem-based defense (Givovich et al., 1992). Since r_m values were calculated based on length of the prereproductive period and fecundity, significant differences in r_m were only detected between three of the *V. darrowi* accessions. Andrewartha and Birch (1954) argued that r_m is the only statistic that adequately represents the physiological qualities of an organism relative to its capacity of increase. Greater variability in aphid resistance within a population of *V. darrowi* may have been detected by calculating r_m values for *I. pepperi* (as was done for between population variability), rather than by measuring the mean total number of aphids after 20 d.

Due to their antioxidant capacity, considerable attention has been paid to the fruit phenolics of *Vaccinium* spp. (Häkkinen and Törrönen, 2000; Taruscio et al., 2004; Ayaz et al., 2005). *Vaccinium* leaf and stem phenolics have received less attention. To our knowledge, this study is the first quantitative description of chlorogenic acid and quercetin derivatives in leaves and stems of *V. darrowi* and *V. corymbosum* cv. Elliott. Witzell et al. (2003) quantified temporal variation in phenolic acids and flavonoids in leaves and stems of boreal bilberry, *Vaccinium myrtillus* L. Similar to our study, Witzell et al. (2003) detected quantitative differences in phenolic profiles between leaves and stems, with higher concentrations generally occurring in the leaves. Furthermore, Witzell et al. (2003) also found a chlorogenic acid derivative (tentatively identified as 5-*O*-caffeoylquinic acid) to be the most abundant compound in leaf samples, while quercetin derivatives were the most abundant flavonols in leaf and stem tissues.

Secondary metabolites, including phenolics, can serve as host-recognition cues and feeding stimulants for monophagous or oligophagous aphids (Montgomery and Arn, 1974; Joerdens-Roettger, 1979; Takemura et al., 2006). As theorized by Fraenkel (1959), the presence of such secondary metabolites can drive the evolution of arthropods from polyphagy to monophagy, whereby the compounds are no longer detrimental but instead, act as beneficial cues. As previously noted, *I. pepperi* is monophagous on *Vaccinium* spp. (Elsner and Kriegel, 1989). Thus, the phenolic acids and flavonol glycosides extracted and identified from the sympatric species *V. corymbosum* may provide host-recognition cues for *I. pepperi*. As previously noted, 5-caffeoylquinic acid represented 83% of the phenolic acids extracted from leaves of *V. corymbosum*. Due to the lack of coevolutionary history between *I. pepperi* and *V. darrowi*, unique or novel ratios of phenolics present in *V. darrowi* may have contributed to resistance.

The localization of secondary metabolites within plant tissues has greater implications for resistance against phloem-feeding insects than other feeding guilds. Phenolic compounds are typically stored in vacuoles or specific storage tissues (Harborne, 1967; Harborne and Grayer, 1993), but they may diffuse into the phloem in their glycoside form (Macleod and Pridham, 1965; Mullin, 1986; Peng and Miles, 1991). If present mainly in the mesophyll, such compounds may be encountered during probing, resulting in the deterrence of phloem feeders (Calatayud et al., 1994). For example, the phenolics

procyanidin, *p*-hydroxybenzaldehyde, and dhurrin deter *Schizaphis graminum* (Rondani) from feeding on resistant sorghum cultivars (Dreyer and Jones, 1981, Dreyer et al., 1981). If localized in the phloem, nutritional deficiencies may occur, resulting in reduced developmental rates and/or fecundity (Givovich et al., 1992). *Aphis fabae* (Scopoli) exhibits reduced fecundity on cowpea, *Vigna unguiculata* L. Walp., lines with elevated quercetin glycoside levels (Lattanzio et al., 2000). Similarly, Calatayud et al. (1994) found that three flavonoid glycosides (rutin, kaempferol glycoside-1, and kaempferol glycoside-2) and cyanogenic glycosides were translocated in the phloem of cassava, *Manihot esculenta* Crantz, and consumed by the mealybug, *Phenacoccus manihoti* Matt. Ferr.

While a direct cause and effect relationship between phenolics and plant resistance to insects is not well documented (Smith, 2005), phenolic compounds play at least a partial role in resistance to several aphid pests (Hedin and Waage, 1986). Furthermore, while identification of the specific mechanism of resistance may not be crucial for the development of resistant cultivars, such information can allow for the utilization of phenotypic or genotypic characters, thereby, facilitating breeding or selection programs (Smith, 2005). For instance, if resistance is chemically based, concentrations of allelochemicals could potentially provide a diagnostic characterization of resistant germplasm. Metabolic profiling can predict levels of plant resistance to insects (Brennan et al., 1992; Brignolas et al., 1998; see Smith, 2005), thereby aiding plant breeders in improving the resistance levels of selected cultivars (Andersson et al., 1980; Hedin et al., 1991). Concentrations of isochlorogenic acid in roots of lettuce, *Lactuca sativa* L., predict resistance levels to the lettuce root aphid, *Pemphigus bursarius* L. (Cole, 1987). Similarly, Lattanzio et al. (2000) found a positive correlation between the flavonoid glycoside content of cowpea, *V. unguiculata*, and aphid resistance.

Vaccinium darrowi and other wild *Vaccinium* spp. represent useful sources of genes providing resistance to abiotic and biotic stresses (Etzel and Meyer, 1986; Meyer and Ballington, 1990; Stretch et al., 2001). The current study characterized variability in aphid resistance and constitutive phenolics exhibited within and between populations of the wild blueberry *V. darrowi*. Several *V. darrowi* accessions were identified as being particularly disruptive to offspring production by *I. pepperi*, which resulted in poor population fitness. As a correlation was not detected between aphid performance and constitutively expressed chlorogenic acid or quercetin derivatives, additional studies should be conducted to assess the role of induced defenses following herbivory by *I. pepperi*. Phloem constituents should also be considered. Offspring resulting from crosses between *V. darrowi* and *V. corymbosum* are currently being screened for *I. pepperi* resistance in an attempt to identify molecular markers associated with insect resistance. Marker-assisted selection of plants based on genotype rather than chemotype could expedite the screening process of advanced *Vaccinium* selections.

Acknowledgments This paper is dedicated to Professor Sridhar Polavarapu (1961–2004), who served as Extension Entomologist for the Philip E. Marucci Center for Blueberry and Cranberry Research and Extension, Rutgers-The State University of New Jersey for 10 years. We thank Michael Reding (Horticultural Insects Research Lab, USDA-ARS, Wooster, OH) and Cesar Rodriguez-Saona (Marucci Center, Rutgers University, Chatsworth, NJ) for reviewing earlier drafts of this manuscript. This work was supported in part by USDA-CSREES 2005-34155-15749 and the New Jersey Agri. Exp. Station project # NJ31912.

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J Chem Ecol (2007) 33:731–747
DOI 10.1007/s10886-007-9253-x

The Distribution of Two Major Iridoids in Different Organs of *Antirrhinum majus* L. at Selected Stages of Development

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Received: 5 September 2006 / Accepted: 1 January 2007 /
Published online: 2 March 2007
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Abstract Two iridoid glucosides isolated from leaves of *Antirrhinum majus* L. were identified as the known compounds antirrhinoside and antirrhide. Plants grown hydroponically demonstrated that antirrhinoside is present in all plant organs including the roots. In contrast, antirrhide is found only in leaves. Furthermore, both iridoids were identified in the main stem axillary leaves and leaves on the lateral branches. The highest concentrations of antirrhinoside were found in the main and lateral stems as well as the buds and flowers. As leaves age, for both cultivars, the levels of antirrhinoside drop significantly, and there is a corresponding increase in antirrhide. In spite of the different genetic backgrounds of the two cultivars, the overall distribution of the iridoids was similar during vegetative and flowering development. Radiolabeling of recently expanded axillary leaves with $^{14}CO_2$ showed that both antirrhinoside and antirrhide were prominently labeled in the laminar tissue. However, only ^{14}C-antirrhinoside was recovered in the subtending petiole tissue, consistent with the suggestion that it is a phloem mobile compound.

Keywords *Antirrhinum majus* · Iridoid glucosides · Plant development · ^{14}C labeling · Optimal · Defense theory

Introduction

Iridoids are a class of terpenoids that occur in plant families of the subclass Asteridae (Jensen, 1992), and over 800 structures have been identified (El-Naggar and Beal, 1980; Boros and Stermitz, 1990, 1991; Grayson, 1996). The function of iridoids in plants has largely been attributed to defense against herbivory by insects (Bowers and Puttick, 1988, 1989), but they also have phytotoxic (Pardo et al., 1998) and antifungal (Marak et al., 2002) activities as well

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as medicinal value (Baruah et al., 1998; Tasdemir et al., 2005). Concentrations of iridoids can reach up to 20% dry weight of new leaves (Bowers et al., 1992). However, the functions of relatively few iridoid glycosides have been investigated. For example, the function of antirrhinoside, an iridoid glucoside commonly found in many species of the Scrophulariaceae, is unknown. In *Asarina scandens*, antirrhinoside is a phloem mobile compound (Gowan et al., 1995). These authors hypothesize that the ability to transport antirrhinoside within the plant to growing sinks could function as a defense against herbivory. However, antirrhinoside may also play a role in plant osmoregulation (Voitsekhovskaja et al., 2006). In a study of another species in the same genus, *Asarina barclaiana*, antirrhinoside comprised 39% of the total carbon transported in the phloem. It was also noted that apoplastic loading of this compound, as well as sucrose, could contribute to regulation of the osmotic pressure during long distance transport of assimilates (Voitsekhovskaja et al., 2006).

In a species of a related genus, *Antirrhinum majus*, Scarpati et al. (1968), Scarpati and Guiso (1969) and Guiso and Scarpati (1969) isolated and identified the iridoid glucosides, antirrhinoside, antirrhide, and 5-*O*-β-glucosyl antirrhinoside from the aerial parts of the plant. Since then, work has mainly focused on the biosynthesis of antirrhinoside (Breinholt et al.,1992; Damtoft et al., 1993, 1995), and we are aware of only one recent study (Høgedal and Mølgaard, 2000), in which the authors investigated the seasonal and diurnal variation in concentrations of iridoids in two field-grown *A. majus* cultivars.

Recently, *A. majus*, like *Arabidopsis thaliana* and *Petunia* spp., has been used as a model system for linking plant molecular genetics with plant architecture and leaf development (Reinhardt and Kuhlemeier, 2002). The natural variation in *A. majus* has been exploited by breeders to generate cultivars with different growth habits for both the bedding plant and cut flower markets (Ball Redbook, 2003). Many cultivars have been bred specifically for the cut flower market, and they are classified broadly into four climatic response groups (Groups I–IV). These vary in their response to key environmental conditions such as day length, light intensity, and temperature (Rogers, 1992).

In *A. majus*, the two major iridoids that represent 90 to 98% of the extractible iridoid glucosides are antirrhinoside and antirrhide (Høgedal and Mølgaard, 2000). These authors found that antirrhinoside decreased in leaf tissue after flowering, but that there was an increase in the concentration of antirrhide. In a field study, these authors were the first to indicate that there may be changes in the patterns of iridoid accumulation in *A. majus* with respect to seasonal and diurnal cycles in the leaf canopy. A more detailed analysis, under controlled environmental conditions, of iridoid concentration at specific leaf position/age as well as in other developing organs such as the stem, flower, and the root tissue is warranted.

The primary purpose of the present study was to determine the distribution of two major iridoids in the different organs of *A. majus* L. at selected stages of vegetative and flowering development. Thus, the major iridoids were first purified and spectroscopically identified. The profiles of the two iridoids among the major organs including the roots were determined for two contrasting F_1 hybrid cultivars (GI and GIV) used by the industry for cut flower production (Rogers, 1992). Potomac Ivory White (GIV) is a cultivar that is recommended for production under summer conditions where high light, long days, and warm temperatures predominate. Maryland Ivory White (GI) is a cultivar better suited for fall and winter when shorter days, lower light, and temperature conditions are prevalent during greenhouse production. Both cultivars were grown under controlled environmental conditions to the flowering stage in hydroponic conditions so that the root tissue as well as the shoot tissue could be examined during selected stages of vegetative and flowering development. Finally, we sought to determine whether or not it is likely that either of the major iridoids in *A. majus* are translocated within the plant through the use of ^{14}C labeling experiment on mature leaves.

Methods and Materials

Instruments

Final purification of compounds for NMR and quantitative analyses by high-performance liquid chromatography (HPLC) was performed on an Agilent model 1100 HPLC equipped with a degasser, quad pump, autosampler, and diode array display (DAD) (Agilent Technologies Canada Inc., Mississauga, ON, Canada). The column used was a CapCell Pak AG 120 C18 (4.6×250 mm, 5 μ particle size) (Shiseido Co. Ltd., Japan), and the HPLC had ChemStation software. The ^{1}H and ^{13}C NMR spectra of 2.0 mg each of the purified compounds in D_2O were obtained with a Bruker (Bruker Spectrospin, Milton, ON, Canada) AV-700 equipped with an UltraShield Plus magnet and cryoprobe in the Department of Chemistry, McMaster University, Hamilton, ON, Canada. A Micromass Quattro Ultima triple quadrupole mass spectrometer was used to obtain mass spectra.

For determining the ^{14}C partitioning in the fed leaf and petiole, a Beckman Intruments Inc. 118 solvent module with a refractive index detector (Beckman Instruments Inc. Altex division) and a LKB Broma 2111 fraction collector were used. The column used was a Supelcosil™ LC-NH_2 amino (4.6×250 mm, 5 μ particle size; Sigma-Aldrich, Canada Inc.). Scintillation counts of the fractions collected were obtained on a model 1414 liquid scintillation counter (Wallac Oy, Turku, Finland). All solvents used were HPLC grade.

Plant Material Used for Bulk Extraction Snapdragon F_1 hybrid seeds of *A. majus* L. cultivars Potomac White Ivory and Maryland White Ivory were provided by PanAmerican Seed™ (Chicago, IL, USA) and were sown and grown in a glass greenhouse in Guelph, ON, Canada (43.3° L.N.). Seeds were sown in a soilless growth medium (Pro-mix GPX®; Premier Horticulture Ltée/LTD Rivière du Loup, QC, Canada) in "128 cavity plug trays" (Landmark Co. Plastic, Akron, OH, USA). Germination took place on a greenhouse misting bench at 22/15°C day/night temperature regime, under natural light conditions. After 3–4 wk, snapdragon seedlings were transplanted to 1.8 l containers filled with aerated nutrient solution as described by Johnstone et al. (2005). To exclude light from the nutrient solution, which would promote the growth of algae, the containers were inserted into a larger black plastic container and its lid covered with black-on-white plastic sheeting, white side upward. Hydroponic single-plant units were positioned in a growth chamber with a 12-hr photoperiod and provided 350±50 μmol m^{-2} $s^{-1}$of photosynthetically active radiation (PAR) at plant canopy height. Day/night temperatures were approximately 23.5/18°C. Plants were fertilized with a commercial hydroponic formulation (1.15 g of 6:11:35 N–P–K supplemented with 0.85 g of $CaNO_3$ per liter, Plant Products Ltd., Brampton, ON, Canada), with a pH of 6.0, and electrical conductivity (EC) of 1.9 mS/cm. Each main stem was supported by a galvanized wire stake (gauge 12). Seedlings received a half-strength nutrient solution for 5 d after transplant. Thereafter, seedlings received full-strength nutrient solution, and the solution level was maintained during the experiment. Both cultivars have been grown successfully in our laboratory to commercial flower maturity (Ortiz-Uribe et al., 2005).

Bulk Extraction and Purification of Major Iridoids from Leaf Tissue Axillary and lateral leaf tissue for bulk extraction was taken from plants of various ages grown over the period of June–Sept. 2005 in growth cabinets at the University of Guelph. Fresh leaf tissue (108 g) from "Potomac" was frozen in liquid N_2, stored at −80°C, freeze-dried, ground to a fine powder (12.88 g), and extracted (in a glass column 6.0×36.0 cm) sequentially with 150 ml hexane, 150 ml ethyl acetate, and 100 ml MeOH. Fractions were dried as much as possible

under a stream of N_2, frozen at −80°C, and then freeze-dried. Dried fractions were weighed and found to be: hexane 0.386 g, EtOAc 0.441 g, and MeOH 2.412 g. The MeOH extract contained high concentrations of the two iridoids by HPLC analysis and was subsequently fractionated on a glass column (2.0×30.0 cm), which had been packed with 23.0 g of chromatographic silica (Fisher 100–200 mesh) suspended in 100 ml $CHCl_3$:MeOH:H_2O 6:3:1 and then equilibrated with the same solvent. The *A. majus* MeOH extract (502 mg) was dissolved in 3.0 ml MeOH and loaded onto the top of the column. By using the above solvent system, the chlorophyll and carotenoids were washed off, whereas the iridoids were retained (as determined by HPLC analysis). The solvent system was then changed to 100% MeOH, and fraction 2 containing the iridoids was collected. Fractions were rotary evaporated to dryness, taken up in 3.0 ml MeOH, dried as much as possible under N_2, frozen at −80°C, and freeze-dried. Fraction 1 weighed 105.5 mg, whereas fraction 2 weighed 385.9 mg. Fraction 2 (100.4 mg) was dissolved in 1.0 ml of MeOH and streaked onto two cellulose 20.0×20.0 cm, 250 μ Avicell microcrystalline cellulose plates (Analtech Inc.) 2.0 cm from the bottom of the plates, allowed to dry, and placed in a TLC tank with *n*-butanol:acetic acid:H_2O 4:1:5 as the mobile phase. Plates were removed after the solvent front had reached 2.0 cm from the top. After removal, plates were allowed to dry, and a 0.5-cm strip on the sides of the plates was sprayed with diphenylamine/aniline/H_3PO_4 spray (Markham, 1982). The side of the plate was heated with a heat gun to visualize the bands. Two main bands were observed at R_f 0.344 (bright blue, C2, later identified as antirrhinoside) and 0.50 (light green, C3, later identified as antirrhide) under UV light (254 nm, Model UVGL-58, Mineralight® Lamp). Using the borders of these bands on the sprayed portion of the plate as a guide, the rest of the plate was marked with a razor blade, and the unsprayed band areas were scraped off and placed in 15 ml plastic centrifuge tubes to which 10 ml of MeOH were added. After vigorous shaking for 15–30 sec, the tubes were centrifuged at 3,000 rpm for 3.0 min, the supernatants were removed, filtered (Whatman 0.2 μm nylon syringe filters), rotary evaporated to dryness, taken up in 1.0 ml MeOH, and checked for purity by HPLC. C2 and C3 were still found not to be totally separated and were replated and treated as above to yield 18.6 mg C2 and 5.3 mg of C3. A further 109.2 mg and 109.7 mg of fraction 2 were plated as above to give standards for use in the HPLC analysis of the distribution of the iridoids within the cultivars and for other experiments.

Mass Spectrometry and NMR Identification Electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode was used, and the following conditions were employed; cone 35 V, capillary 3 kV, desolvation gas 400 l/hr (±200), desolvation temp 200°C. Loop injections were carried out with 50 μl/min flow of MeOH/H_2O. The following NMR experiments were carried out to elucidate the structures: 1D: ^1H and ^{13}C, 2D: DQF-COSEY, HSQC, and HMBC.

Sampling and Preparation of Plant Tissue for Quantitative HPLC Analysis There are two types of leaves in *A. majus*, axillary and lateral. Axillary leaves are those directly attached to the main stem. They develop first. Lateral leaves are attached to lateral stems that branch from the main stem above the axillary. Axillary leaves and lateral stems originate from slightly enlarged areas of the main stem, defined as nodes. Axillary leaves were harvested from the fourth, eighth, and 12–14th node from the base. Lateral leaves harvested were the first pair of leaves on the lateral stem at the fourth and eighth node of the main stem. Plant organs were harvested 44, 54, 61, 68, 75, 82, 89, 96, and 106 days after sowing (DAS) for the Potomac cultivar, and 44, 54, 61, 68, 75, 82, and 89 DAS for the Maryland cultivar. Three replicate plants per cultivar were harvested at the same time of the day between 13:00–14:30 hr.

Leaves were removed at the stem, wrapped in aluminum foil, immediately frozen in liquid N_2, stored in a −80°C freezer, and freeze-dried. Nonleaf tissue was separated according to organ type (i.e., stem, root, etc.) and then treated as the leaves were.

Before HPLC analysis, the lyophilized whole stems were broken into several pieces and ground to a fine powder, from which a subsample was taken for homogenization and HPLC analysis. Flowers were separated from the green sepals, pooled for each plant, and ground to a fine powder, from which a subsample was taken. For the roots, a sample was taken from the medial portion of the freeze-dried root mass, and for the leaves, the apical portion was taken. Samples of all plant organs were weighed and placed in 1.5 ml eppendorf tubes to which 1.0 ml of MeOH and two stainless steel beads (Montreal Biotech, Inc., QC, Canada) were added. We generally extracted between 10 and 25 mg of dry plant material, but in extreme cases, such as the when the lateral stems were just forming, as little as 2 mg may have been available for analysis. Tubes were placed in a Retsch MM300 tissue homogenizer (Cole-Parmer Canada, Inc., Anjou, QC, Canada) for 5.0 min at 30 cycles per second, removed and centrifuged at 12,000 rpm for 2.0 min. Supernatants were pipetted off, and filtered through 0.2 μm nylon syringe filters (Whatman International Ltd., Maidstone, UK) into HPLC sample vials.

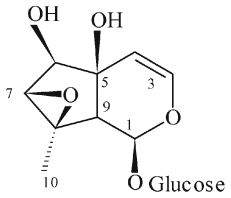
HPLC Conditions For quantification of the iridoids, the solvent system was initially isocratic employing 3% ACN in H_2O after Høgedal and Mølgaard (2000) with a flow rate of 1.0 ml/min and an elution period of 15 min. However, it was found that by using 6% ACN in H_2O the elution time could be reduced to 10.0 min with adequate separation of the iridoid compounds in samples. Samples were scanned (200–600 nm), and the wavelength used for quantitation was 205 nm. For determining the ^{14}C partitioning in the fed lamina tissue and petiole, the solvent system was 75% ACN in H_2O with a flow rate of 1.0 ml/min.

Steady-state $^{14}CO_2$ Labeling Plants were grown under the conditions described above. When plants had 14–18 axillary leaves, they were used in the labeling experiment. $^{14}CO_2$ steady-state labeling of the most physiologically mature leaves (fourth or fifth node) was conducted for 3 hr by using an open-flow system described previously by Jiao and Grodzinski (1996). Detailed descriptions of the leaf cuvette system, the steady state gas exchange, and the $^{14}CO_2$ labeling protocol were similar to Leonardos et al. (2003). The specific activity of the $^{14}CO_2$ gas among experiments varied from 0.28 to 0.36 kBq μmol^{-1} C depending on the leaf photosynthesis rate. However, during each feeding, the specific activity of the $^{14}CO_2$ was constant.

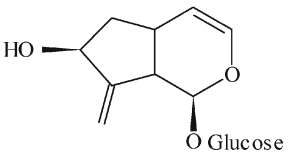
Before the 3-hr feed period, vegetative seedlings were transferred to the growth chamber at the beginning of the photoperiod where they were allowed to acclimate and reach a steady rate of photosynthesis for 1 hr. The growth chamber conditions were as follows: the light level supplied by 1000 W metal halide lamps (Sylvania GTE, Toronto, ON, Canada) was set at 1,000 $\mu mol\ m^{-2}\ s^{-1}$ PAR at the leaf surface; the temperature was at 25±1°C and a relative humidity of 50% was maintained inside each cuvette by passing the gas stream through a gas bubbler in a controlled-temperature water bath. The mean (N=8) photosynthetic rate was 16.5±0.48 $\mu mol\ C\ m^{-2}\ s^{-1}$. At the end of the labeling period, the fed leaf was separated into two parts: the $^{14}CO_2$-fed laminar tissue was inside the cuvette, whereas the subtending petiole was positioned outside the feeding cuvette and, therefore, not exposed to the $^{14}CO_2$. Both lamina and petiole tissue were wrapped in aluminum foil, immediately frozen in liquid N_2, stored in a −80°C freezer, and freeze-dried. The fed leaf area and petiole dry weights were obtained. Each sample was placed in a 1.5-ml eppendorf tube to which 1 ml of methanol (MeOH) and two stainless steel beads (Montreal

Table 1 ^{1}H and ^{13}C NMR data for antirrhinoside and antirrhide in D_2O. Samples for purification and analysis were prepared from bulk extractions as outlined in Methods and Materials

	Antirrhinoside	
	δ_H	δ_C
Aglycone		
1	5.47 (1H, d, *J*= 6.24)	93.6
3	6.43 (1H, d, *J*= 6.26)	141.7
4	4.93 (1H, d, *J*= 6.28)	105.6
5	-	73.0
6	4.05 (1H, d, *J*= 2.17)	75.4
7	3.57 (1H, d, *J*= 2.2)	64.9
8	-	63.7
9	2.47 (1H, d, *J*= 6.24)	50.6
10	1.46 (3H, s)	15.6
Glucose		
1'	4.77 (1H, d, *J*= 8.14)*	97.9
2'	3.31 (1H, t, *J*= 8.07)	72.0
3'	3.47 (1H, t, *J*= 9.23)	75.0
4'	3.37 (1H, t, *J*= 9.09)	69.0
5'	3.40-3.45 (1H, m)	75.7
6'a	3.89 (1H, dd, *J*= 4.31/12.36)	60.1
6'b	3.70 (1H, dd, *J*= 5.87/12.36)	



	Antirrhide	
Aglycone		
1	5.53 (1H, d, *J*= 2.67)	94.6
3	6.16 (1H, dd, *J*= 2.03/6.35)	138.3
4	4.85 (1H, br. d, *J* = 7.14)	107.2
5	2.81 (1H, t, *J*= 7.24)	27.6
6a	2.01 (1H, dd, *J*= 7.0/12.34)	37.9
6b	1.70 (1H, m)	
7	4.60 (1H, m)	72.2
8	-	151.0
9	3.06 (1H, t-like)	42.9
10a	5.31 (1H, t, *J*= 2.55)	110.5
10b	5.28 (1H, t, *J*= 2.53)	
Glucose		
1'	4.77 (1H, d, *J*= 8.1)*	97.7
2'	3.27 (1H, t, *J*= 9.5)	72.2
3'	3.47 (1H, m)	75.1
4'	3.37 (1H, t, *J*= 9.57)	69.1
5'	3.46 (1H, m)	75.7
6'a	3.89 (1H, dd, *J*= 2.25/12.36)	60.2
6'b	3.69 (1H, dd, *J*= 3.66/12.38	



Abbreviations: br.=broad, s=singlet, d=doublet, dd=doublet of doublets, t=triplet, t-like = triplet-like, m=multiplet
**J* may not be accurate because of damping of HDO peak.

Biotech, Inc., QC, Canada) were added. Samples were homogenized in a Retsch MM300 tissue homogenizer for 5 min at 30 cycles per sec, removed, and passed through a 0.2 μm filter (Whatman International, Ltd., Maidstone, UK). Individual sugars and iridoids were separated by HPLC at ambient temperature. Effluent was collected, and the radioactivity in the individual sugars and iridoids was determined by liquid scintillation counting.

Statistical Analyses As the cultivars differ in their development, plants were grouped according to their developmental stage (vegetative, budding, or flowering) for comparisons. A two-way analysis of variance (ANOVA) was carried out for plant organ type and cultivar as the main effects (PROC GLM) with SAS® (2005) statistical software package. One-way ANOVAs with Student–Newman–Keuls means difference tests were used to determine differences in iridoid concentrations in different plant tissues by using the (PROC GLM, Means SNK).

Results

Identification and Quantification of the Two Major Iridoids in A. majus In Table 1, the proton and carbon assignments, and coupling constants are given along with the corresponding structures for antirrhinoside and antirrhide obtained from bulk extraction

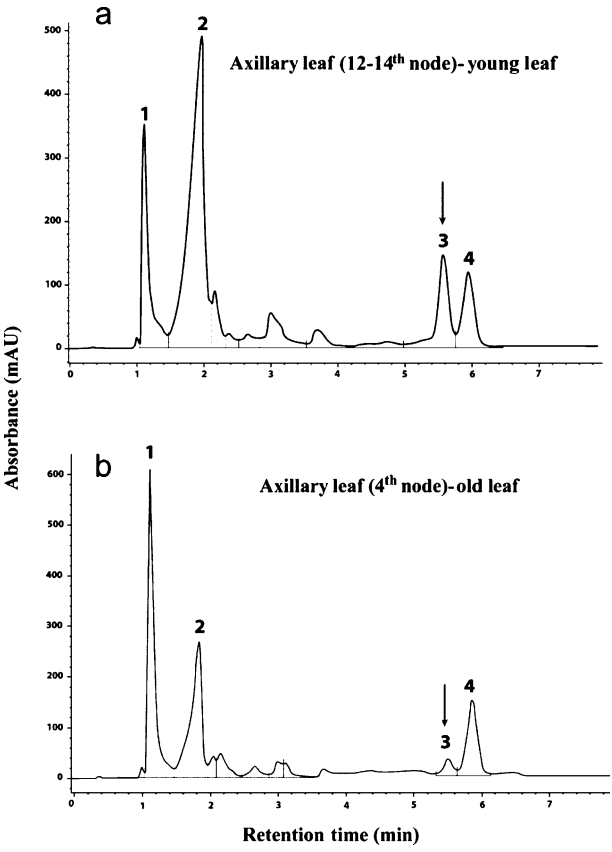


Fig. 1 (a) Chromatogram of MeOH extract from an axillary leaf taken from the 12th node showing the levels of antirrhide relative to antirrhinoside. 1=unidentified carotenoid, 2=mixture of unidentified non-iridoid compounds, 3=antirrhinoside, 4=antirrhide. (b) Chromatogram of axillary leaf taken from the fourth node of the same plant

Table 2 Two-way analysis of variance for the two major iridoids, antirrhinoside and antirrhide, by organ and cultivar

Source	Degree of freedom (*df*)	Type III SS	Mean square	*F* value	*P*>*F*
Antirrhinoside					
Organ	9	658,751.18	73,194.57	53.48	<0.001
Cultivar	1	6,918.30	6,918.30	5.05	0.025
Organ×cultivar	9	28,374.02	3,152.66	2.30	0.016
Antirrhide					
Organ	4	5,737.17	1,434.29	3.71	0.006
Cultivar	1	3,123.84	3,123.84	8.07	0.005
Organ–cultivar	4	839.75	209.93	0.54	0.705

of the leaves. The ES-MS data yielded a primary *m*/*z* ion corresponding to the hydrated molecular ion of antirrhinoside at *m*/*z* 380.3 [362+H_2O(18)], and at *m*/*z* 348.0 [330+H_2O(18)] for the hydrated molecular ion of antirrhide.

The modified HPLC method of Høgedal and Mølgaard (2000), as described in "Methods and Materials," was used to quantify antirrhinoside and antirrhide levels in different tissues. For example, the HPLC trace (Fig. 1a,b), shows graphically how young axillary leaves near the top of the plant canopy (e.g., 12th node) had higher levels of antirrhinoside than did the older leaves (e.g., fourth node) within the canopy. Calibration curves were constructed by using the purified and identified compounds at concentrations of 1.0, 0.5, 0.25, 0.125, and 0.0625 mg/ml injected into the HPLC giving curves with R^2 values of 0.9991 and 0.9998 for antirrhinoside and antirrhide, respectively.

Comparison of Iridoid Concentrations in Plant Organs Between Cultivars The two-way ANOVA with plant organ and cultivar as the main effects showed that the main effects were significant for both antirrhinoside and antirrhide, but that the interaction was significant only for antirrhinoside (Table 2). This indicates that the pattern of antirrhinoside allocation in plant tissues differs between the two cultivars. Figure 2 shows the pattern of allocation of the major iridoids antirrhinoside and antirrhide for the two cultivars Potomac and Maryland at two specific stages of development, the budding and flowering stage. Maryland develops and flowers faster than Potomac, and has fewer axillary leaves. A comparison of the two cultivars at the same morphological stage indicates that the only significant difference is for budding and flowering tissue, which has only antirrhinoside (Fig. 2c,d). There were no significant differences in the levels of antirrhinoside or antirrhide between the two cultivars in the vegetative stage (data not shown). At other stages, when the concentrations of individual compounds were examined, statistically significant differences are apparent. During budding, Maryland (N=5) had more antirrhinoside in the axillary leaf at node 4 than Potomac (N=7), but Potomac had more of this iridoid in the lateral stems (Fig. 2c) than did Maryland. During flowering, Maryland (N=8) had more antirrhinoside in the 12th node leaf, whereas Potomac (N=7) had more in the flowers (Fig. 2d). However, during budding Potomac had more antirrhide in the axillary leaves than did Maryland (Fig. 2e), but there were no differences for the lateral leaves. During the flowering stage, Potomac had more antirrhide than Maryland in axillary leaves at nodes 8 and 12, and node 4 for the laterals (Fig. 2f).

Comparison of Iridoid Concentrations in Plant Organs Within Each Cultivar It is apparent from Tables 3 and 4 that younger leaf tissue, both axillaries and laterals, initially have relatively high levels of antirrhinoside, but this gradually declines as the leaves age.

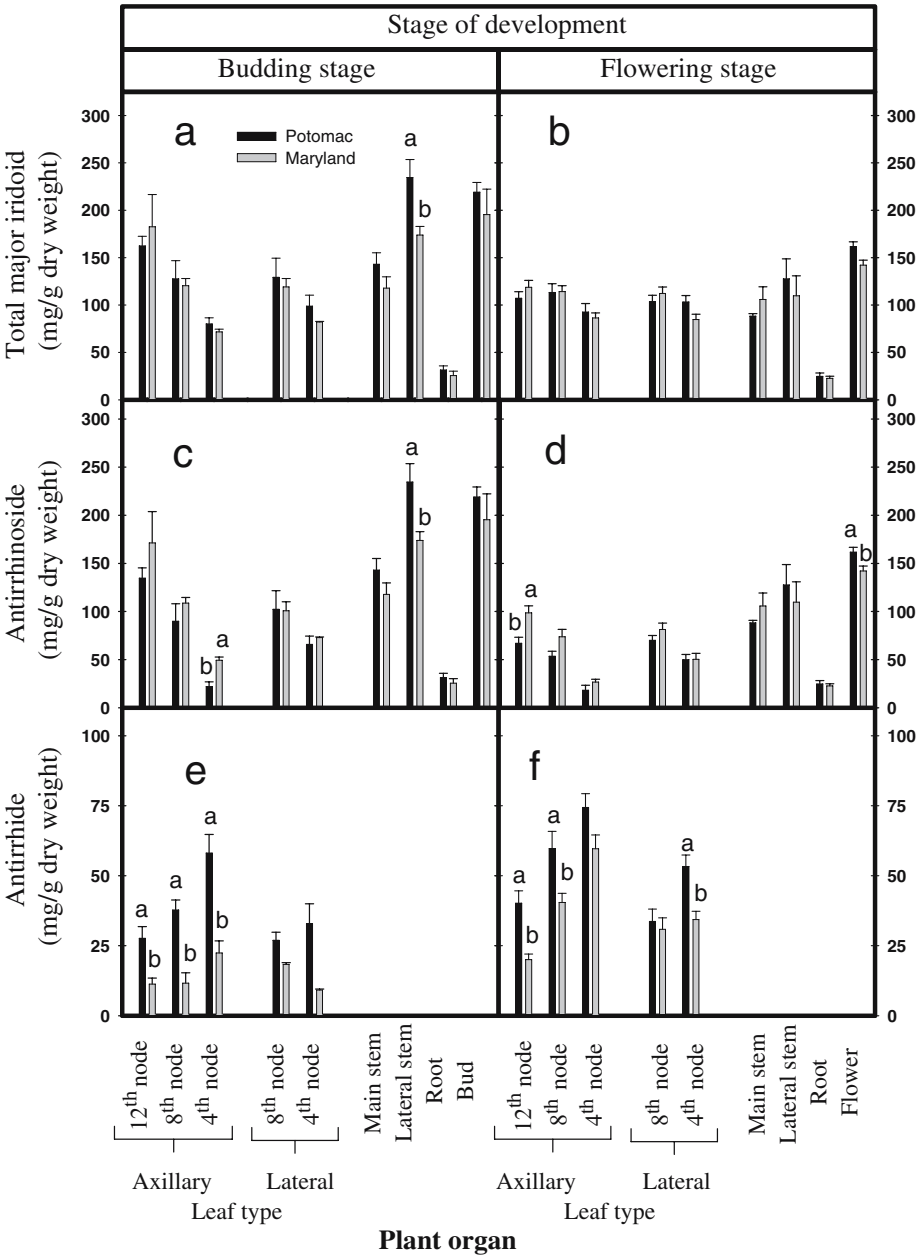


Fig. 2 Distribution of the total major iridoids (a, b), antirrhinoside (c, d), and antirrhide (e, f) among plant organs sampled at two stages of flowering development (budding and flowering) in two snapdragon cultivars (Potomac and Maryland Ivory White). Bars represent means with SE and letters indicate significant differences ($P<0.05$)

Table 3 The distribution of the two major iridoids, antirrhinoside and antirrhide (mg/g dry weight), in different organs of *A. majus* cv. Potomac Ivory White at selected stages of vegetative and flowering development

Days after sowing	Leaves					Other organs			
	Axillary leaf			Lateral leaf		Stem		Root	Bud *
	Fourth node	Eighth node	12–14th node	Fourth node	Eighth node	Main	Lateral		Flower
Antirrhinoside concentration (mg/g DW)									
44	74.7^{a} (16.9)	–	–	–	–	67.1^{a} (5.4)	–	50.4^{a} (6.0)	–
54	73.0^{a} (18.8)	–	–	–	–	54.4^{a} (8.0)	–	28.5^{a} (3.8)	–
61	77.7^{c} (10.2)	144.8^{b} (22.8)	–	–	–	90.0^{c} (9.1)	218.3^{a} (10.5)	35.5^{d} (2.5)	–
68	45.6^{e} (8.5)	140.7^{c} (7.5)	189.4^{b} (4.9)	92.8^{d} (9.1)	–	111.7^{d} (5.2)	237.0^{a} (10.4)	40.6^{e} (2.7)	–
75	39.6^{c} (1.1)	114.5^{b} (16.5)	142.5^{b} (27.0)	71.2^{c} (0.8)	128.1^{b} (5.5)	140.3^{b} (5.7)	211.6^{a} (5.1)	32.2^{c} (8.2)	–
82	19.0^{d} (3.7)	81.2^{cd} (36.7)	116.4^{cd} (7.1)	68.5^{cd} (15.7)	151.5^{bc} (42.0)	153.6^{bc} (28.2)	261.0^{a} (30.0)	28.9^{d} (9.1)	231.2^{ab*} (9.9)
89	11.1^{d} (1.9)	57.4^{cd} (13.0)	120.0^{bc} (14.3)	52.5^{cd} (11.5)	84.6^{cd} (9.2)	112.9^{bc} (14.2)	176.5^{ab} (50.0)	31.4^{cd} (5.9)	200.9^{a*} (15.0)
96	11.3^{d} (1.3)	60.1^{c} (3.7)	66.7^{c} (11.2)	51.9^{c} (3.5)	68.3^{c} (10.5)	83.2^{c} (1.7)	121.4^{b} (23.3)	20.8^{d} (4.0)	155.9^{a} (9.2)
106	26.6^{c} (11.0)	49.9^{c} (11.6)	58.9^{c} (4.0)	51.5^{c} (12.1)	69.7^{c} (7.5)	93.5^{bc} (4.1)	143.7^{ab} (47.8)	23.3^{c} (3.1)	171.2^{a} (3.4)
Antirrhide concentration (mg/g DW)									
44	10.3 (0.6)	–	–	–	–	nd	–	nd	–
54	7.5 (2.1)	–	–	–	–	nd	–	nd	–
61	22.8^{a} (4.5)	5.6^{a} (0.8)	–	–	–	nd	nd	nd	–
68	30.3^{a} (4.4)	10.1^{b} (2.2)	10.4^{b} (0.7)	14.5^{b} (1.0)	–	nd	nd	nd	–
75	50.1^{a} (11.0)	28.4^{ab} (1.3)	16.4^{b} (0.53)	25.2^{ab} (2.9)	12.9^{b} (1.8)	nd	nd	nd	–
82	66.0^{a} (11.6)	45.7^{ab} (5.2)	28.8^{b} (5.7)	27.3^{b} (11.8)	20.8^{b} (5.5)	nd	nd	nd	nd
89	61.6^{a} (1.9)	43.7^{ab} (9.5)	38.0^{ab} (4.6)	47.6^{ab} (3.7)	30.1^{b} (1.3)	nd	nd	nd	nd
96	74.3^{a} (7.9)	53.5^{b} (3.47)	29.1^{c} (3.3)	44.1^{b} (4.0)	24.9^{c} (3.3)	nd	nd	nd	nd
106	80.0^{a} (6.7)	64.9^{ab} (14.8)	51.3^{ab} (1.7)	63.8^{ab} (1.3)	43.4^{b} (6.1)	nd	nd	nd	nd

Means with SE in brackets. Means within a row that are followed by the same superscripted letter are not significantly different ($P>0.05$)
DW = dry weight; – = tissue not developed; nd = not detected

Table 4 The distribution of the two major iridoids, antirrhinoside and antirrhide (mg/g dry weight) in different organs of *A. majus* cv. Maryland Ivory White at selected stages of vegetative and flowering development

Days after sowing	Leaves					Other organs			
	Axillary leaves			Lateral leaves		Stem		Root	Bud *
	Fourth node	Eighth node	12–14th node	Fourth node	Eighth node	Main	Lateral		Flower
Antirrhinoside concentration (mg/g DW)									
44	108.7^{a} (11.9)	–	–	–	–	113.3^{a} (25.4)	–	60.0^{a} (11.5)	–
54	72.5^{a} (12.4)	–	–	–	–	70.3^{a} (24.8)	–	18.8^{a} (1.5)	–
61	59.1^{c} (2.0)	97.3^{b} (5.8)	–	–	–	92.2^{b} (4.3)	177.9^{a} (11.1)	33.7^{d} (4.1)	–
68	43.9^{de} (0.7)	115.0^{bc} (6.2)	171.3^{ab} (32.6)	72.9^{cde} (0.6)	100.9^{cd} (5.5)	133.2^{abc} (13.0)	177.8^{ab} (15.1)	19.0^{e} (2.8)	195.4^{a*} (26.9)
75	35.2^{c} (5.0)	100.8^{abc} (8.5)	117.6^{ab} (17.9)	62.9^{bc} (10.4)	86.2^{abc} (3.3)	145.7^{a} (22.7)	151.2^{a} (5.1)	29.6^{c} (2.8)	147.7^{a} (14.1)
82	19.0^{c} (1.3)	57.4^{bcd} (8.5)	79.5^{b} (1.7)	38.9^{bc} (5.9)	88.3^{ab} (18.9)	66.3^{bc} (5.6)	132.8^{a} (28.2)	17.4^{c} (0.7)	132.1^{a} (3.4)
89	25.9^{ef} (3.0)	63.2^{cd} (4.6)	105.3^{b} (6.8)	45.5^{de} (9.6)	69.7^{c} (7.5)	105.6^{b} (6.5)	45.4^{de} (1.0)	21.2^{f} (3.1)	146.5^{a} (6.2)
Antirrhide concentration (mg/g DW)									
44	10.3 (0.7)	–	–	–	–	nd	–	nd	–
54	9.1 (2.4)	–	–	–	–	nd	–	nd	–
61	13.6^{a} (5.6)	8.5^{b} (0.9)	–	–	–	nd	nd	nd	–
68	27.2^{a} (2.3)	16.8^{b} (2.8)	11.3^{bc} (2.1)	9.2^{c} (0.4)	18.3^{b} (0.6)	nd	nd	nd	nd
75	53.7^{a} (5.3)	29.6^{b} (3.4)	22.6^{b} (1.3)	27.4^{b} (2.5)	22.4^{b} (5.8)	nd	nd	nd	nd
82	54.8^{a} (12.7)	44.0^{ab} (3.3)	20.0^{b} (5.0)	31.7^{ab} (0.81)	26.8^{ab} (2.3)	nd	nd	nd	nd
89	70.5^{a} (3.1)	47.7^{b} (3.7)	18.4^{c} (2.8)	43.0^{b} (3.3)	43.4^{b} (6.1)	nd	nd	nd	nd

Means with SE in brackets. Means within a row that are followed by the same superscripted letter are not significantly different ($P>0.05$)
DW = dry weight; – = tissue not developed; nd = not detected

Conversely, antirrhide is found at low concentrations in young leaves, but this increases with leaf age. However, the concentration of these iridoids in different plant tissues varies dramatically. For example, at 75 DAS, Potomac has high levels of antirrhinoside in stems and young (developing) leaves (Table 3). What is particularly striking is the magnitude of differences in antirrhinoside concentrations in different organs of the same plant at a given time. For example, at 82 DAS, the buds and lateral stems of Potomac have concentrations of antirrhinoside that are close to an order of magnitude greater than that of the roots and fourth node axillary leaves (Table 3). The Maryland cultivar (Table 4) shows a similar pattern except earlier in development (68 DAS).

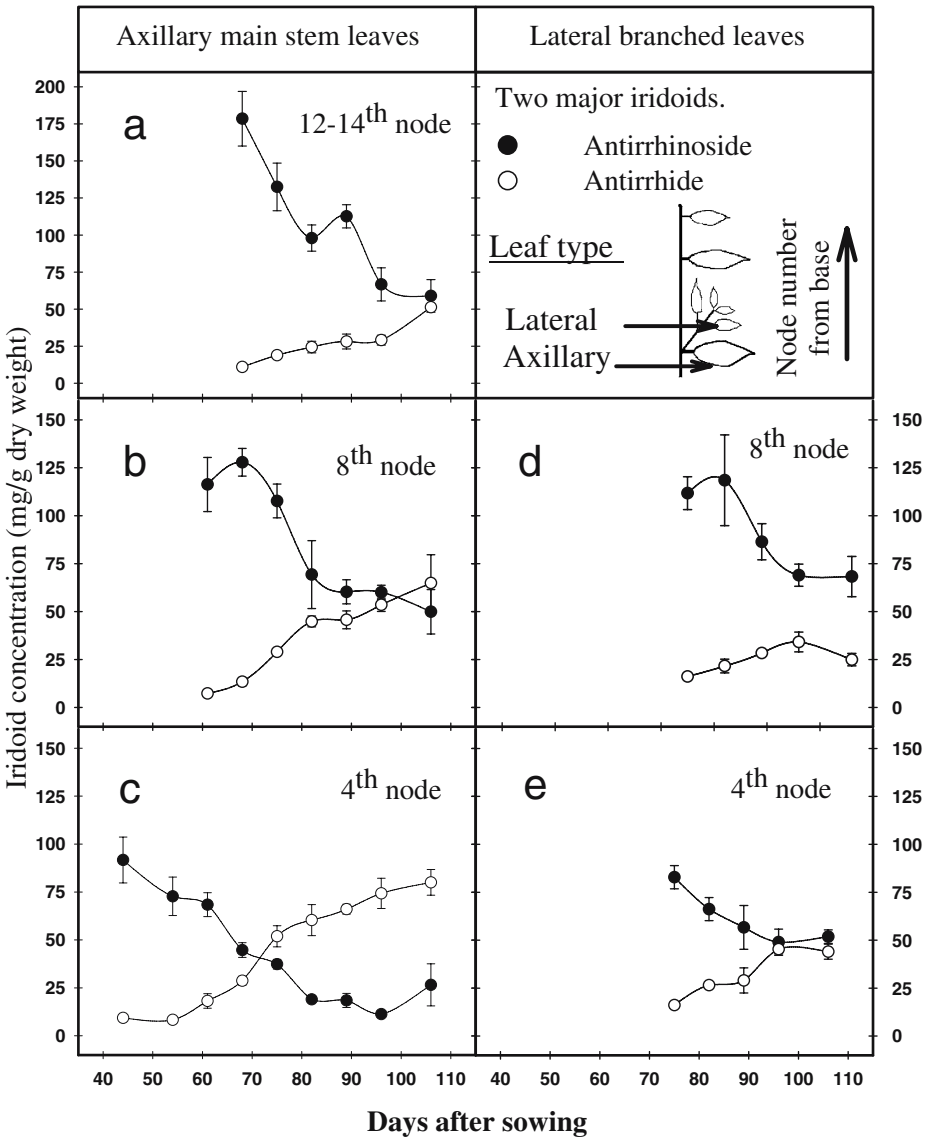


Fig. 3 Distribution of the total major iridoids, antirrhinoside, and antirrhide in axillary (a, b, c) and lateral leaves (d, e) analyzed by pooling data from the two snapdragon cultivars (Potomac and Maryland Ivory White) at similar stages of shoot development. Data represent the means and SE of at least six samples except for days 96 and 106 where only three samples were available from Potomac Ivory White. Bars represent SE of the mean

With regard to other plant organs and concentration of antirrhinoside, no pattern as observed for the leaves was apparent. The roots, except for the first sampling period, had consistently low values for both cultivars, whereas the lateral stems had consistently high levels. Bud tissue had the highest level of antirrhinoside compared to all other tissues for both cultivars. At the time of final harvest, flowers had the highest level of antirrhinoside compared to all other plant organs. Antirrhide was not detected in nonleaf tissue.

Table 5 Labeling pattern of antirrhinoside and antirrhide in the fed lamina tissue and the connecting petiole tissue of the lamina of *A. majus* that was labeled with $^{14}CO_2$ for 3 hr under steady-state conditions as described in "Methods and Materials"

	Percent label relative to total label		Percent label relative to sucrose label	
	Fed lamina tissue	Petiole	Fed lamina tissue	Petiole
Antirrhinoside	22.1 (1.5)	14.5 (1.3)	44.7 (4.4)	47.2 (4.7)
Antirrhide	8.0 (0.01)	nd	16.4 (2.0)	nd
Iridoid total	30.1	14.5	61.1	47.2

Labeling patterns for the two major iridoids were expressed as a percentage of the total label recovered in the tissue and also relative to a primary photoassimilate, sucrose (after Gowan et al., 1995). Data are the means with SE in brackets of at least eight leaves, four leaves for each cultivar
nd = not detected

Iridoid Distribution in the Leaf Canopy In *A. majus*, the leaf canopy is composed initially only of axillary leaves. However, as the plant matures, lateral branches and leaves develop, and at the time of flowering the lateral leaves are the dominant photosynthetic organs. As seen above there are differences in the rate of development of the two cultivars, but they have similar patterns of accumulation of the two major iridoids in the leaf tissue. When the accumulation of leaf iridoids is combined, based on the same DAS, the general pattern of concomitant decrease in antirrhinoside and increase in antirrhide is striking (Fig. 3). Young axillary leaves initially have high levels of antirrhinoside, but as they age the amounts drop to much lower levels at all node positions (Fig. 3a,b,c). A similar trend is seen in the laterals as the canopy develops (Fig. 3d,e). In all leaves, there was also a corresponding increase in the amount of antirrhide produced as the leaves matured (Fig. 3a–e).

Partitioning of ^{14}C in the Fed Leaf and Petiole Tissue Table 5 shows the labeling pattern of antirrhinoside and antirrhide in the laminar and petiole tissues of a physiologically mature, expanded, source leaf following a 3-hr period of steady-state photosynthesis in the presence of $^{14}CO_2$. There were no significant differences in the labeling patterns between cultivars (data not shown). The data for the two cultivars when combined show that antirrhide was found only in the labeled leaf, whereas labeled antirrhinoside was present in both the lamina and the petiole of each cultivar. On average, the two major iridoids contained 30% of the total fixed ^{14}C in the laminar tissue. In relation to sucrose in the laminar tissue, these two compounds accounted for ca. 60% of the labeling. In the laminar tissue, the antirrhinoside pool contained three times as much ^{14}C label as did the antirrhide pool. In the petiole, labeled antirrhinoside, the only labeled iridoid, accounted for 15% of the total newly fixed ^{14}C recovered or 47% of the ^{14}C-photoassimilates in relation to sucrose.

Discussion

For antirrhinoside, our ^{1}H and ^{13}C chemical shifts agree closely with those published using either D_2O (Kitagawa et al., 1973; Bianco et al., 1981; Franzyk et al., 1999) or CD_3OD (Ercil et al., 2004; Al-Rehaily et al., 2006) as an NMR solvent, with the exception that we were able to resolve most of the glucose protons (Table 1). For antirrhide, the only published ^{1}H spectrum that we are aware of is that of Kitagawa et al. (1973) in which only

ranges for the chemical shifts are estimated. Therefore, in Table 1, we provide the full assignment of the chemical shifts as well as coupling constants. The ^{13}C spectrum for antirrhide in Table 1 is in general agreement with that of Otsuka (1993) in CD_3OD.

Following the identification of the two major iridoids, we quantified their distribution in all organs of *A. majus* L. during vegetative and flowering development. The two cultivars were sampled over a period of 45 (Maryland) and 62 (Potomac) days, respectively, under controlled environmental conditions. With respect to leaf iridoid concentration, we found that, regardless of cultivar, the concentration of antirrhinoside and antirrhide changed with leaf position, suggesting that leaf age may be a determinant of not only the amount, but also the type of iridoid present (Tables 3 and 4). Specifically, axillary and lateral leaves show a change in the pattern of iridoid accumulation where antirrhinoside decreases with leaf age and antirrhide increases.

A rise in one iridoid and fall in another, which is dependant on leaf age, has been found for *Plantago lanceoleta* (Plantaginaceae), where older leaves had no iridoids or predominantly aucubin, and younger leaves primarily or only catalpol (Klockars et al., 1993). These authors suggest that as aucubin is a precursor to catalpol it is rapidly converted in the newest leaves and, therefore, undetectable. As leaves age, catalpol may be transported out, broken down, or aucubin may no longer efficiently be converted to catalpol. However, antirrhide is not known to be a precursor to antirrhinoside (Damtoft et al., 1993, 1995), and it may be in our study that antirrhinoside was transported out of older source leaves to developing sinks, such as younger leaves, buds, and flowers. There is little information in the literature about either the amount or types of iridoids found in flowers (but see Mead and Stermitz, 1993 and Song et al., 2006). This is, to our knowledge, the first study that documents the presence of antirrhinoside in flowers of any species in the Scrophulariaceae.

Antirrhinoside is phloem mobile in other species within the Scrophulariaceae (Gowan et al.,1995; Voitsekhovskaja et al., 2006). The absence of antirrhide in nonleaf tissue may indicate that it is synthesized in the leaves but not transported within the plant as antirrhinoside is. Our study demonstrates the different allocation of these two major iridoids within the different plant organs of *A. majus*, and we also document for the first time the presence of antirrhinoside in the root tissue. Antirrhide is not found in the roots of either cultivar.

Overall, the total leaf amounts of antirrhinoside and antirrhide are higher than those reported by Høgedal and Mølgaard (2000). However, in their seasonal study, field-grown plants were subjected to changing weather conditions, and the collected tissue samples were pressed and dried at 40°C for 24 hr. In contrast, our plants were grown under hydroponic conditions where nutrients were nonlimiting and temperature and light were controlled. In addition, samples in our study were frozen in liquid N_2, stored at −80°C, and lyophilized before analysis.

Although there is lack of information on the potential of antirrhinoside as an allelochemical, its distribution and concentration within *A. majus* cultivars follow what would be expected based on Optimal Defense (OD) theory (McKey, 1974). This theory proposes that the allocation of defense compounds within a plant is related to the fitness value of the plant parts and the probability that they will be attacked by herbivores. In *A. majus*, the highest levels of antirrhinoside were found in the buds and flowers, followed by young vs. older leaves, and finally by roots, which have the lowest levels as predicted by OD theory. Although antirrhinoside is not known to be deterrent to insect herbivores, it is sequestered by two species of moths that are aposematic as larvae and adapted to feeding on high antirrhinoside-containing plants (Boros et al., 1991). However, antirrhinoside is not found in the cryptic adults of the two species. Furthermore, catalpol, which has a structure

similar to antirrhinoside, significantly reduced the growth of the generalist gypsy moth (*Lymantria dispar* L.) larvae when fed at 0.18% in artificial diet (Bowers and Puttick, 1988).

It is also known that iridoids deter feeding by generalist insect herbivores, but stimulate feeding in specialists (Bowers and Puttick, 1988), and also are induced in response to fungal infection (Marak et al., 2002). Iridoids are present in higher concentrations in younger leaves of *Plantago lanceolata* (Klockars et al., 1993; Stamp and Bowers, 1994), and it has been hypothesized that this tissue is more active photosynthetically and thus costly for the plant to lose to herbivores or infection (McKey, 1974; Ohnmeiss and Baldwin, 2000). If one of the functions of antirrhinoside is as a defensive compound, then given the above it is not unexpected that there is more in developing sink tissues such as young leaves, buds, and flowers.

The ^{14}C labeling data (Table 5) show that labeled antirrhide is found only in the lamina tissue, whereas labeled antirrhinoside is found both in the petiole and lamina tissue. These observations parallel those of two other species in the Scrophulariaceae, *A. scandens* (Gowan et al., 1995) and *A. barclaiana* (Voitsekhovskaja et al. 2006) where antirrhinoside is phloem mobile. In Table 5, the amount of label relative to that in sucrose (after Gowan et al., 1995) and to the total label recovered in the tissue at the end of 3 hr is given. This indicates that almost 15% of the total labeled carbon in the petiole is recovered in the antirrhinoside pool. In contrast, antirrhinoside represented 39% of the total carbon transported for the species for *A. barclaiana* (Voitsekhovskaja et al., 2006). Both our ^{14}C labeling experiment and the general pattern of distribution of antirrhide in the laminar tissue only, suggest that it is not phloem mobile. Our data, however, do not provide conclusive evidence that antirrhinoside is made only in the leaves and that this is the only iridoid glucoside translocated throughout the plant, as it is possible that antirrhinoside could have been synthesized in sink tissues from exported labeled sucrose.

At this time, it is not known with any certainty what the function of antirrhinoside is in *A. majus* and what, if any, relationship it may have with antirrhide. The high concentration of antirrhinoside in the growing sinks and its probable movement indicates that one of its roles may be as an osmoregulator that maintains water balance within the plant (Voitsekhovskaja et al., 2006). However, its concentration and distribution also suggests that it may be important in defense. The finding that in the oldest axillary leaves (fourth node) antirrhide can be present at up to three times the concentration of antirrhinoside indicates that it probably has a function within those tissues, but what that function is and why it is not translocated as antirrhinoside may be, is unclear.

Acknowledgments We thank Dr. Don Hughes of the Department of Chemistry, McMaster University, Hamilton, Ontario, Canada for obtaining the NMR spectra and George Lin for help in the laboratory. We also thank the following funding agencies: National Sciences and Engineering Research Council Canada, Flowers Canada (Ontario) Ltd. and Ontario Ministry of Agriculture, Food and Rural Affairs. This work was also supported by an Ontario Centers of Excellence ETech Cooperative Research Award held by R.R.C.

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J Chem Ecol (2007) 33:749–765
DOI 10.1007/s10886-007-9257-6

Inter- and Intraspecific Variation in Floral Scent in the Genus *Salix* and its Implication for Pollination

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Received: 12 July 2006 / Revised: 20 November 2006 / Accepted: 29 January 2007 /
Published online: 2 March 2007
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Abstract The floral scent composition of 32 European and two Asian *Salix* L. species (Salicaceae) was analyzed. Intra- and interspecific variation was compared for a subset of 8 species. All *Salix* species are dioecious and floral scent was collected from both male and female individuals by using a dynamic headspace MicroSPE method, and analyzed by GC-MS. A total of 48 compounds were detected, most of them being isoprenoids and benzenoids. Commonly occurring compounds included *trans*-β-ocimene, *cis*-β-ocimene, benzaldehyde, D-limonene, α-pinene, *cis*-3-hexenyl aceatate, linalool, 1,4-dimethoxybenzene, and β-pinene. Two compounds, 1,4-dimethoxybenzene and *trans*-β-ocimene, were responsible for most of the interspecific variation. In a subset of eight extensively sampled species, six had a characteristic floral scent composition; half of the pairwise species comparisons confirmed significant differences. In three of these eight species, intraspecific variability could be explained by sex differences. Variation in *Salix* floral scent may provide specific signals that guide pollinators and thus contribute to the reproductive isolation of compatible and cooccurring species.

Keywords Dioecy · Floral scent · GC-MS · Intraspecific variation · Interspecific variation · *Salix* · Salicaceae

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Introduction

The genus *Salix* L., composed of approximately 400 to 500 species (Skvortsov, 1999), has an almost worldwide distribution, but occurs predominantly in temperate to arctic regions of the northern hemisphere. In Central Europe, about 40 species occur and many are sympatric.

From the taxonomic point of view, *Salix* is a problematic genus with difficulties delimiting many species because of high morphological variability (Argus, 1997; Skvortsov, 1999) and supposed widespread hybridization and introgression (Mosseler, 1990). There are several, in some parts dissentient, phylogenetic classifications of the genus available, all based on morphological characters (Dorn, 1976; Argus, 1997: American species; Skvortsov, 1999: Eurasian species). Because it is the most comprehensive for Eurasian species, the classification of Skvortsov (1999) is used here. Skvortsov (1999) divided *Salix* into three subgenera (Chamaetia, Salix, and Vetrix), each with several sections.

Normally, willow species are dioecious with flowers arranged in catkins. The plants show traits of insect as well as wind pollination. Stiff erect catkins and the availability of nectar fit with insect pollination, whereas small flower size, the absence of a perianth, and the predominant flowering early in spring before leaf unfolding, match with the wind pollination syndrome. Hence, the importance of either mode of pollination in *Salix* is controversial (Karrenberg et al., 2002). Nevertheless, most species are thought to be mainly entomogamous, though in certain species wind contributes to some degree to pollination (Argus, 1974; Sacchi and Price, 1988; Vroege and Stelleman, 1990; Totland and Sottocornola, 2001; Karrenberg et al., 2002). Flowers of both sexes are visited by a wide variety of insects, including Diptera (Vroege and Stelleman, 1990; Tollsten and Knudsen, 1992; Totland and Sottocornola, 2001), Hymenoptera (van der Werf et al., 1982; Vroege and Stelleman, 1990; Tollsten and Knudsen, 1992; Totland and Sottocornola, 2001), Lepidoptera (Vroege and Stelleman, 1990; Totland and Sottocornola, 2001), Coleoptera (Vroege and Stelleman, 1990), and occasionally birds (Kay, 1985). Flower-visiting animals are rewarded with easily accessible pollen and nectar (male flowers) or solely with nectar (female flowers). In most cases, it is not clear to what extent particular flower visitors contribute to effective pollination (van der Werf et al., 1982).

From hybridization experiments (Argus, 1974; Salick and Pfeffer, 1999; Palme et al., 2003) and analyses of natural populations (Mosseler and Papadopol, 1989; Mosseler and Zsuffa, 1989; Rechinger, 1992; Triest et al., 1999), it is clear that many willow species are able to hybridize. For example, more than 50 different hybrid combinations are known from the approximately 30 species that occur in Germany (see Rothmaler, 2002). However, how often hybridization occurs under natural conditions and what role introgressive hybridization plays (Dorn, 1976; Triest et al., 1997; Salick and Pfeffer, 1999; Totland and Sottocornola, 2001) is still a matter of discussion. Our understanding of the nature and efficiency of isolating mechanisms in sympatric compatible willow species, e.g., phenological differentiation (Argus, 1974; Dorn, 1976; Mosseler and Papadopol, 1989) or incongruity (Argus, 1974; Mosseler, 1989; Adler, 2000) is still incomplete. Floral scent is one trait that might function as a reproductive isolating mechanism in entomogamous species by guiding pollinating insects to specific species. However, there are few studies available that compare floral scent across several species within a genus to test this hypothesis. The only study in *Salix* that investigates floral scent variability within and among species was done by Tollsten and Knudsen (1992). They studied two sympatrically occurring, insect-pollinated species, *Salix caprea* and *Salix cinerea*, and both displayed relatively similar floral scent profiles. They concluded that floral scent does not promote reproductive isolation between these two species, resulting in the frequently observed hybridization.

In dioecious plants, such as *Salix* species, it is essential that pollen is transported from male to female flowers and that pollinators fly among them. Tollsten and Knudsen (1992) hypothesized that the floral scent of males and females should not differ within a species; otherwise, pollinators could learn to associate the scent of either gender with its rewards, resulting in preference for one sex. Indeed, they found no difference in scent between male and female flowers within either *S. caprea* or *S. cinerea*, suggesting that pollinating insects cannot discriminate among the sexes of these species.

In the present study, the floral scent of 34 willow species was analyzed by using a dynamic headspace MircoSPE method. The main objectives were to provide an overview of scent production in this interesting genus, with respect to its pollination biology, and to determine intrageneric, interspecific, and intraspecific variation. Based on our results, we discuss the potential of floral scent patterns as reproductive isolation barriers, and as cues for pollen collecting bees to discriminate between male and female individuals.

Methods and Materials

Plant Material Among the 34 species of *Salix* studied, 23 had been planted in the Ecological–Botanical Garden Bayreuth, Germany (EBG). Details on the geographic origin of these plants are listed in Table 1. All other species studied either grew wild in the EBG and/or at sites near Bayreuth. Thirty-two of the studied species are native to Europe; two occur naturally in Asia only (*Salix babylonica*, *Salix gracistyla*).

For 26 species, only a few individuals were available (Table 1), and floral scent could be collected only from one or two male and/or female specimens. For 8 species, several plants of both sexes were available and at least two male and three female specimens were sampled for variability among sexes within these eight species, and to compare intraspecific with interspecific variability. Five out of these eight extensively sampled species (*S. caprea*, *S. cinerea*, *Salix fragilis*, *Salix triandra*, and *Salix viminalis*) grow wild at sites near Bayreuth. Specimens of the other three species—*Salix bicolor*, *Salix myrsinifolia*, and *Salix repens*—have been planted at the EBG and have different geographical origins each (Table 1).

Volatile Collection Floral scent samples were collected from individuals in full bloom in the field from March to May 2005. Scent samples were taken during the day (10:00–17.00) by using a dynamic headspace method. For each individual plant, one twig with four to ten flowering catkins, depending on catkin size, was enclosed for 10 min in an oven bag (Nalophan). The emitted floral scent was subsequently trapped for 2.5 min in a microtube filled with absorbent (3 mg of a 1:1 mixture of Tenax-TA 60–80 and Carbotrap 20–40) by using a membrane pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany). Airflow rate during volatile collection was 200 ml min^{-1}. After sampling, the microtubes were stored in a freezer (at −20°C) until analysis.

Gas Chromatography and Mass Spectrometry (GC–MS) The samples were analyzed on a Varian Saturn 3800 gas chromatograph (GC) fitted with a 1079 injector, and a Varian Saturn 2000 mass spectrometer (MS). A ZB-5 column (5% phenyl polysiloxane, length 60 m, inner diameter 0.25 μm, film thickness 0.25 μm, Phenomenex) was used for the analyses. Microtubes were inserted via Varians' Chromatoprobe into the GC injector. The injector vent was opened (1/20) and the injector heated at 40°C to flush any air from the system. After 2 min, the split vent was closed and the injector heated at 200°C min^{-1}, then held at 200°C for 4.2 min, after which the split vent was opened (1/20) and the injector cooled down.

Table 1 Species, systematic position (according to Skvortsov, 1999), number of samples from males (M) and females (F), location of sampled plants, and geographic origin (data as far as available) of willow plants studied

Species	Abbreviation	Section	M	F	Location	Geographic Origin[a]
Subgenus *Chamaetia*						
S. glauca L.	*S. gla*	Glaucae		1	EBG[b]	N (west), Grotli/Geiranger, 1,250 m
Subgenus *Salix*						
S. triandra L.	*S. tri*	Amygdalinae	2	3	Wild[c]	D, Bavaria, Bayreuth, 365 m
S. pentandra L.	*S. pen*	Pentandrae		1	EBG	D, Saxony-Anhalt, Quedlinburg, 455 m
S. alba L.	*S. alb*	Salix	2		Wild	D, Bavaria, Bayreuth, 340 m
S. fragilis L.	*S. fra*	Salix	3	3	Wild	D, Bavaria, Bayreuth, 340 m
S. babylonica L.	*S. bab*	LSubalbae		1	Wild	D, Bavaria, Bayreuth, 365 m
Subgenus Vetrix						
S. arbuscula L.	*S. arb*	Arbuscella	1		EBG	N (south), Kongsvoll, 1,000 m
S. arbuscula L.		Arbuscella	1		EBG	CH, St. Gallen, Gamperfin, 1,320 m
S. bicolor Willd.	*S. bic*	Arbuscella	2		EBG	F (east), Vogesen, Hohneck, 1,200 m
S. bicolor Willd.		Arbuscella		1	EBG	No data
S. bicolor Willd.		Arbuscella	1		EBG	N (west), Gjevil see, Oppdal, 600 m
S. bicolor Willd.		Arbuscella		1	EBG	CZ (nord), Tatra, 1,800 m
S. cantabrica Rech. f	*S. can*	Arbuscella		1	EBG	E (north), Kantabrien, Sia Pass, 1,050 m
S. foetida DC.	*S. foe*	Arbuscella	1		EBG	I, Aosta, Gr. St. Bernhard, 2,020 m
S. foetida DC.		Arbuscella		1	EBG	No data
S. eleagnos Scop	*S. ele*	Canae		1	EBG	CH, St. Gallen, Neckertal, 580 m
S. acutifolia Willd.	*S. acu*	Daphnella	1		EBG	No data
S. daphnoides Vill.	*S. dap*	Daphnella	1		Wild	D, Bavaria, Bayreuth 365 m
S. daphnoides Vill.		Daphnella	1		EBG	A, Steiermark, Graz, 440 m
S. daphnoides Vill.		Daphnella		1	EBG	CH, St. Gallen, Sitterufer, 570 m
S. crataegifolia Bertol.	*S. cra*	Glabrella	1		EBG	I, Tuscany, Orto di Donna, 1,450 m
S. glabra Scop.	*S. gla*	Glabrella	1		EBG	CH, Tessin, Val Colla, Fojorina-Nord, 1,650 m
S. hastata L.	*S.has*	Hastatae		2	EBG	CZ (north), Sudeten Mountains, 1,300 m
S. hastata L.		Hastatae	1		EBG	No data
S. caesia Vill.	*S. cae*	Helix	1		EBG	F (southeast), Col de Larche, 1900 m
S. caesia Vill.		Helix		1	EBG	CH, Grisons, Bevers, Ebene, 1,700 m
S. purpurea L.	*S. pur*	Helix	2	1	Wild	D, Bavaria, Bayreuth, 365 m
S. repens L.	*S. rep*	Incubaceae	1		EBG	No data
S. repens L.		Incubaceae	2		EBG	No data
S. repens L.		Incubaceae	1		EBG	PL (east), Brzezno, 200 m
S. repens L.		Incubaceae		2	EBG	DK (south), Bornholm, 30 m
S. repens L.		Incubaceae		1	EBG	N (south), Bergen, 43 m
S. apennina Skv.	*S. ape*	Nigricantes		1	EBG	I, Tuscany, Cisa-Pass, 450 m
S. apennina Skv.		Nigricantes		1	EBG	I, Verona, Apua, Mte Altissimo, 1,300 m
S. mielichhoferi Sauter.	*S. mie*	Nigricantes	1		EBG	A, Salzburgerland, Radstätter Tauern, 1,700 m
S. mielichhoferi Sauter.		Nigricantes	1		EBG	I, Südtirol, Seiseralp, 1,200 m
S. mielichhoferi Sauter.		Nigricantes		1	EBG	A, Steiermark, Tauern, 1,750 m

Table 1 (continued)

Species	Abbreviation	Section	M	F	Location	Geographic Origin[a]
S. myrsinifolia Salisb.	*S. myr*	Nigricantes	1		EBG	CH, St. Gallen, Wattwil, 620 m
S. myrsinifolia Salisb.		Nigricantes	1		EBG	N (west), Gjevil See, 700 m
S. myrsinifolia Salisb.		Nigricantes	1	1	EBG	CH, Grisons, Vorderrhein, 1,500 m
S. myrsinifolia Salisb.		Nigricantes		2	EBG	CH, St. Gallen, Wattwil, 620 m
S. gracilistyla Miq.	*S. gra*	Subviminales	1		EBG	J (cultivated)
S. appendiculata Vill.	*S. app*	Vetrix	1		EBG	CH, Tessin, Airolo, 1,200 m
S. atrocinerea Brot.	*S. atr*	Vetrix		1	EBG	IR (east), Wicklow, Glendalaugh, 600 m
S. atrocinerea Brot.		Vetrix	1		EBG	CH, St. Gallen, Rohrspitz, 400 m
S. aurita L.	*S. aur*	Vetrix	1		Wild	D, Bavaria, Bayreuth, 365 m
S. caprea L.	*S. cap*	Vetrix	3	2	Wild	D, Bavaria, Bayreuth, 365 m
S. cinerea L.	*S. cin*	Vetrix	2	3	Wild	D, Bavaria, Bayreuth, 365 m
S. cinerea L.		Vetrix	1		EBG	CH, St. Gallen, Wattwil, 670 m
S. laggeri Wimm.	*S. lag*	Vetrix	1		EBG	CH, Wallis, Gletschboden, 1,780 m
S. laggeri Wimm.		Vetrix		1	EBG	A, Tirol, Stubei, 1,600 m
S. silesiaca Willd.	*S. sil*	Vetrix	1		EBG	CR (north), Sudeten Mountains, 1,400 m
S. silesiaca Willd.		Vetrix	1	1	EBG	PL (east), W-Tatra, 1,300 m
S. silesiaca Willd.		Vetrix	1		EBG	CR (north), Sudeten Mountains, 1,300 m
S. starkeana Willd.	*S. sta*	Vetrix		1	EBG	S, (east), Jämtland, Tännäs, 20 m
S. helvetica Vill.	*S. hel*	Villosae	2		EBG	CH, Wallis, Grimselpass, 2,040 m
S. helvetica Vill.		Villosae		1	EBG	CH, Wallis, Gletschboden, 1,780 m
S. lapponum L.	*S. lap*	Villosae	1		EBG	CR (north), Sudeten Mountains, 1,400 m
S. lapponum L.		Villosae	1		EBG	CR (north), Sudeten Mountains, 1,300 m
S. viminalis L.	*S. vim*	Vimen	3	3	Wild	D, Bavaria, Bayreuth, 365 m

[a] The geographic origin is described with the shortcut of European countries and m declared the level above sea.

[b] EBG = individuals cultivated in the Ecological-Botanical Garden Bayreuth.

[c] Wild = growing wild in natural habitats.

Electronic flow control was used to maintain a constant helium carrier gas flow of 1.8 ml min^{-1}. The GC oven temperature was held for 7 min at 40°C, then increased by 6°C min^{-1} to 260°C, and held for 1 min at this temperature. Mass spectra were taken at 70 eV with a scanning speed of 1 scan/sec from *m*/*z* 30 to 350.

The GC-MS data were processed with the Saturn Software package 5.2.1. To identify floral scent components, GC-MS spectra were compared to, the NIST 02 and MassFinder 3 databases. Identifications were confirmed by comparison of retention times with published data (Adams, 1995). Identification of some compounds was also confirmed by comparison of mass spectra and retention times with those of authentic standards.

Statistics To determine (semi)-quantitative differences among single samples, we used chord-normalized expected species shared (CNESS) dissimilarity index, ranging between 0 and square root of 2. These semiquantitative comparisons were based on the percentage amount of components. Comparison of the absolute peak areas and the amounts were impractical because emission rate varied extensively both within and among species across individuals and flowering period. In cases where more individuals per species had been sampled, mean relative amounts per species were calculated. The CNESS index was calculated by using the updated version of the Combinatorial Polythetic Agglomeration Hierarchical Clustering (COMPAH) program (Boesch, 1977), provided by Gallagher at UMASS/Boston (http://www.es.umb.edu/edgwebp.htm).

Qualitative differences in floral scent (presence or absence of compounds) among samples were determined by using Sørensen's index of similarity (Sørensen, 1948). RELATE was used (program package Primer, version 5.2.9) to correlate and compare the CNESS with the Sørensen matrix (Kendall correlation coefficient).

We utilized nonmetric multidimensional scaling (NMDS) in the STATISTICA 7 package to identify meaningful dimensions and to visualize both similarities and dissimilarities among individual samples or different species (see Borg and Lingoes, 1987). A stress value is given to calculate how well the particular configuration produces the observed distance matrix. The smaller this value, the better is the fit of the configuration to the reproduced distance matrix (Clarke, 1993).

Analysis of similarities (ANOSIM, one-way design) in the program package Primer (version 5.2.9) was used to test for differences in floral scent among species of subgenera *Salix* and *Vetrix*, and within subgenus *Vetrix* among species of sections *Arbuscella* and *Vetrix*. We used these combinations because too few species were sampled in subgenus *Chamaetia* and the other sections making a statistical test less powerful.

Analysis of similarities (two-way crossed design; factors: species and sex) was further used to test for differences in floral scent among eight species (with five or six individuals sampled), and within these species between male and female individuals.

CNESS dissimilarity matrices were used for all ANOSIM analyses. This test calculates the test statistic R as well as a level of significance. R value ranges between 0 and 1 (−1) and can be interpreted as follows: 1 indicates complete separation of the sample groups (e.g., subgenera), and small values (close to zero) imply no segregation (Clarke and Warwick, 2001).

We used ANOVA as a global test and subsequently the Tukey–Kramer test as a *post hoc* test to compare the mean relative amount of the two most variable scent compounds between species. Normality was tested by using the Kolmogorov–Smirnov test and homogeneity of variances was tested with the Hartley test.

A variance component analysis in the STATISTICA 7 package was utilized to estimate the contribution of single floral scent compounds to the total observed variation (relative amount) between species.

Results

The compounds found in the floral scent samples of 93 willow plants from 34 species, are listed in Table 2. A total of 48 compounds were detected and 43 were identified. Dominant compound classes included isoprenoids and benzenoids, but fatty acid derivates and N-containing compounds were also present. The most commonly occurring compounds were *cis*-

Table 2 Chemical composition of floral scents: occurrence and relative amount of each compound detected in the flower scent of 93 individuals (52 male and 41 female) of 34 *Salix* species

Compound[a]	R_i[b]	Occurrence[c]	Relative Amount[d]					
			Male			Female		
			Median	Quartiles	Min–Max	Median	Quartiles	Min–Max
Isoprenoids								
α-Phellandrene[e]	934	11	0	0–0.25	0–1.41	0	0	0–0.11
α-Pinene[e]	957	29	1.01	0.11–7.37	0–42.34	1.01	0.2–3.57	0–84.46
Camphene[e]	958	10	0	0–0.08	0–3.33	0.23	0–1.61	0–4.79
Sabinene[e]	987	18	0	0–1.18	0–6.67	0	0–0.51	0–4.88
β-Pinene[e]	995	20	0.67	0–3.72	0–26.45	0.16	0–1.6	0–15.54
β-Phellandrene[e]	1,026	9	0	0–0.03	0–1.52	0	0	0–3.33
D-Limonene[e]	1,045	31	1.04	0.29–4.15	0–18.32	0.74	0.12–3.14	0–33.32
cis -β-Ocimene[e]	1,048	33	6.48	3.32–9.59	0–32.50	5.21	3.04–11.94	0–19.78
trans-β-Ocimene[e]	1,058	34	26.76	12.13–45.78	0.59–93.94	21.18	9.21–48.03	0–87.98
γ-Terpinene[e]	1,071	13	0	0–0.53	0–10.28	0	0	0–4.14
trans-Linalool oxide[e]	1,099	2	0	0–1.84	0	–	–	–
Linalool[e]	1,104	25	0.61	0–7.25	0–32.84	3.39	0.1–6.74	0–64.19
Lilac aldehyde A[e]	1,153	17	0.03	0–0.96	0–9.40	0	0–0.11	0–4.26
Camphor	1,153	4	0	0	0–0.76	0	0	0–0.49
Lilac aldehyde B+C[e]	1,163	23	0.19	0–1.24	0–13.91	0.08	0–1.74	0–17.03
Lilac aldehyde D[e]	1,178	13	0	0–0.22	0–7.23	0	0	0–3.56
4-Terpineol	1,191	1	0	0	0–0.59	–	–	–
α-Terpineol	1,202	5	0	0	0–3.45	–	–	–
Lilac alcohol A[e]	1,211	4	0	0	0–3.33	0	0	0–0.17
Lilac alcohol B+C[e]	1,219	5	0	0	0–1.70	0	0	0–0.41
Lilac alcohol D[e]	1,232	3	0	0	0–0.85	0	0	0–0.14
D-Verbenone[e]	1,228	14	0	0–0.76	0–21.01	0	0–0.27	0–32.33
α-Copaene	1,397	5	0	0	0–0.18	0	0	0–3.46
β-Bourbonene	1,407	11	0	0–0.01	0–0.62	0	0	0–9.89
(*E*)-Caryophyllene[e]	1,447	9	0	0	0–5.33	0	0–0.02	0–10.43
E-Geranylacetone[e]	1,336	19	0	0–0.03	0–5.33	0.02	0–0.5	0–5.03
Cubebene	1,334	3	–	–	–	0	0	0–2.47
(*E*,*E*)-α-Farnesene[e]	1,508	15	0.01	0–0.19	0–1.69	0	0	0–0.39
Benzenoids								
Benzaldehyde[e]	982	31	0.78	0.15–1.81	0–10.22	0.23	0–1.61	0–4.79
Benzyl alcohol[e]	1,050	4	0	0	0–3.36	0	0	0–0.96
Phenylacetaldehyde[e]	1,060	3	0	0	0–22.19	0	0	0–0.95
Salicylaldehyde	1,063	4	0	0	0–8.66	0	0	0–0.01
4-Methylbenzylalcohol	1,077	4	0	0	0–0.21	0	0	0–0.5
2-Phenylethanol[e]	1,123	7	0	0	0–1.83	0	0	0–6.31
Veratrole[e]	1,153	11	0	0	0–1.82	0	0	0–18.34
1,4-Dimethoxybenzene[e]	1,175	21	0.5	0–5.78	0–77.36	0	0–2.79	0–74.58
Methyl salicylate[e]	1,208	24	0.54	0–4.39	0–27.94	0.35	0–2.03	0–16.53
N-bearing compounds								
Benzyl nitrile[e]	1,144	5	0	0	0–1.12	–	–	–
Indole[e]	1,254	6	0	0	0–0.08	0	0	0–1.44

Table 2 (continued)

Compound[a]	R_i[b]	Occurrence[c]	Relative Amount[d]					
			Male			Female		
			Median	Quartiles	Min–Max	Median	Quartiles	Min–Max
Fatty acid derivates								
cis -3-hexen-1-ol[e]	860	21	0.08	0–1.81	0–8.4	0.19	0–1.73	0–9.61
cis-3-hexenyl acetate[e]	1,016	28	6.16	0.1–17.05	0–36.79	3.75	0.31–13.99	0–64.69
(*E*)-4,8-Dimethyl-1,3,7-nonatriene[e]	1,118	24	0.34	0–0.82	0–3.37	0.07	0–0.46	0–10.00
4-Oxoisophorone[e]	1,159	16	0	0–0.66	0–7.89	0	0–0.6	0–18.67
Unknown substance								
65, 77, 93, 105, 121, 136	1,033	8	0	0–0.08	0–0.71	0	0	0–0.21
39, 77, 93, 105, 121, 136	1,100	13	0	0–0.33	0–4.58	0	0	0–0.38
39, 65, 79, 91, 107, 122	1,130	22	0,1	0–0.74	0–2.27	0.26	0–0.57	0–1.59
41, 67, 82, 105, 122, 138	1,172	5	0	0	0–4.91	0	0	0–1.61
41, 57, 67, 82, 103, 120	1,204	22	0	0–0.31	0–4.14	0.14	0–0.45	0–13.29

[a] Compounds within classes are listed according to Kovat`s index.

[b] Kovat's retention index.

[c] Number of species where a compound was detected.

[d] Relative proportion (%) of the compounds in the floral scent bouquets of 52 male and 41 female samples.

[e] Identity confirmed by comparison of MS and retention time with those of authentic standards.

and *trans*-β-ocimene (found in 33 and 34 species, respectively), D-limonene (31 species), benzaldehyde (31species), *cis*-3- hexenyl aceatate (28 species), linalool (25 species), 1,4-dimethoxybenzene (21 species), and α- and β-pinene (29 and 20 species, respectively). The number of compounds detected in each species ranged from a low of four in *Salix acutifolia*, and five in *S. silesiaca*, and *S. glauca* to a high of 29 in *S. myrsinifolia*. The scent profiles in all species were dominated by few components only. Dominant compounds reaching on average at least 50% of the total scent mixture within a species were *trans*-β-ocimene (in *S. viminalis*, *S. daphnoides*, *S. repens*, *S. triandra*, *S. apennina*, *S. bicolor*, *S. glabra*, *S. acutifolia*, *S. babylonica*, and *S. gracilistyla*) and 1,4-dimethoxybenzene (in *S. caprea*, *S. atrocinerea*, *S. aurita*, and *S. cinerea*).

Interspecific Variation Comparing the relative amounts of floral scent compounds among all species (by using a variance component analysis), seven compounds explained 94.7% of the total observed variation among the species. Two compounds, 1,4-dimethoxybenzene (35.9%) and *trans*-β-ocimene (32.5%), were responsible for most of the interspecific variation, followed by α-pinene (11.1%), *cis*-3-hexenyl acetate (9.2%), linalool (3.0), D-limonene (1.8%), and D-verbenone (1.2%).

Differences in floral scent composition (relative amounts) among 34 *Salix* species based on the CNESSm=1 index are shown in Fig. 1, using nonmetric multidimensional scaling (stress=0.19). In general, no clear separation of species groups was found. Most species

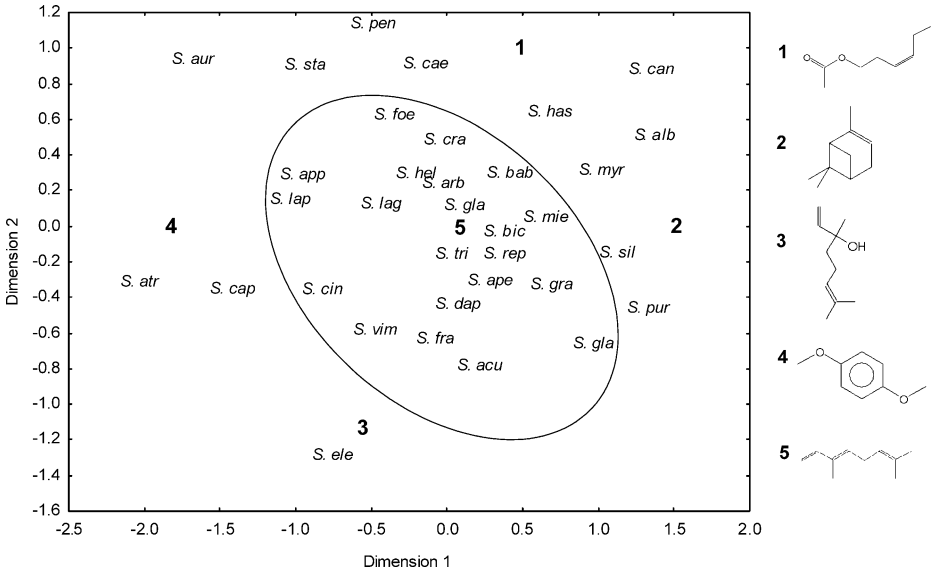


Fig. 1 Nonmetric multidimensional scaling (NMDS) of floral scent profiles of 34 *Salix* species based on the CNESSm=1 index (stress=0.19). The structures and names of the five main compounds: *1 cis*-3-hexenyl acetate, *2* α-pinene, *3* linalool, *4* 1,4-dimethoxybenzene, 5 *trans*-β-ocimene dominating the scent of different species are presented in the figure. The *circle* comprises species with more than 30% relative amount of *trans*-β-ocimene

were more or less evenly distributed, and clear separation of subgroups was hardly possible. Species in the center of the scatter plot were characterized by the emission of high relative amounts of *trans*-β-ocimene, while the amount of this monoterpene was lower in species at the margins. In *S. caprea*, *S. atrocinerea*, *S. aurita*, and *S. cinerea,* high amounts of 1,4-dimethoxybenzene were found. In other species (*S. mielichhoferi*, *S. myrsinifolia*, and *S. silesiaca*), high amounts of α-pinene (25–35%) were detected. High amounts of the green leaf volatile *cis*-3-hexenyl acetate (50–65%) were emitted by *S. starkeana* and *S. pentandra*, and the isoprenoid linalool occurred in large amounts (32%) in *S. eleagnos*.

When analyzing the data qualitatively by using the Sφrensen index, which considers similarity based on the presence or absence of single compounds for comparison and not their relative amount, the results were similar with most species being evenly distributed according to nonmetric multidimensional scaling (stress=0.17), indicating that categorization of species based on scent composition is hardly possible. The CNESS and Sørensen matrices were strongly correlated (RELATE Kendall: R=0.181; P<0.001), and the results of both analyses were generally consistent. Therefore, the NMDS representing the Sørensen matrix is not displayed here.

Analyses of floral scent composition of species from the two subgenera *Salix* (N=5) and *Vetrix* (N=28) revealed no differences between these subgenera (CNESS, ANOSIM: R=−0.035; P=0.66). However, within the *Vetrix* subgenus, significant differences between species of section *Arbuscella* (N=4) and *Vetrix* (N=8) were found (CNESS, ANOSIM: R=0.274; P<0.005). A variance component analysis revealed *cis*-3-hexenyl acetate and 1,4-dimethoxybenze as the main variable compounds between these two sections. A relatively high amount of *cis*-3-hexenyl acetate was found in section *Arbuscella* and 1,4-dimethoxybenze in section *Vetrix*.

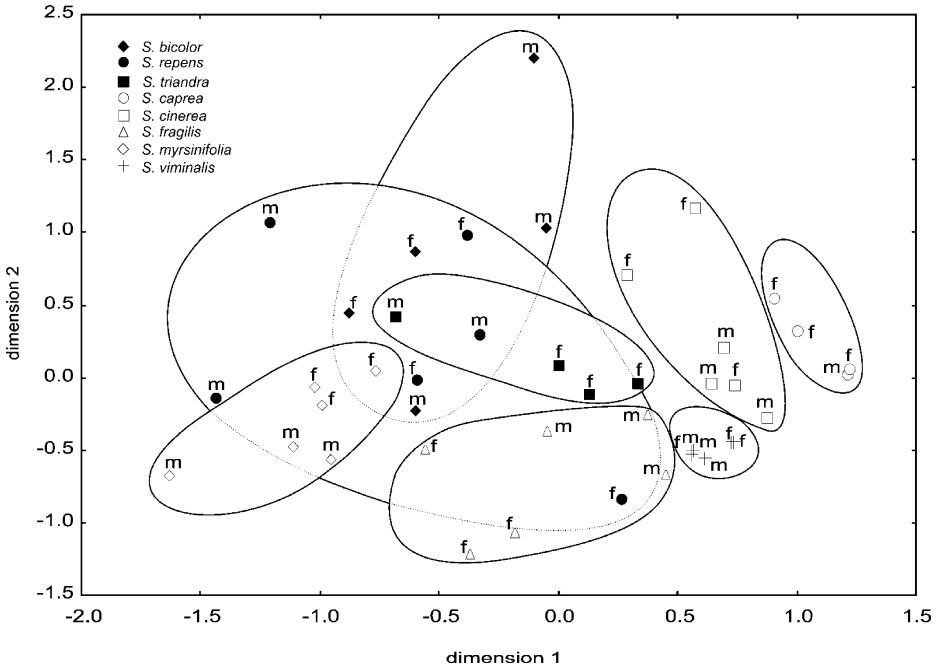


Fig. 2 Intraspecific comparison of floral scent between males (*m*) and females (*f*): nonmetric multidimensional scaling (NMDS) of eight *Salix* species based on the CNESSm=37 index (stress=0.18)

The variability of floral scent among and within the eight extensively sampled species is shown in Fig. 2. Variability within species (based on all samples from both sexes) was lower than variability among species (ANOSIM: R=0.598; P<0.001). When ignoring *S. bicolor* and *S. repens*, the two relatively variable species, the remaining six species had characteristic floral scent profiles, as revealed by grouping of individual samples of each taxon together in a NMDS analyses (Fig. 2). Out of 28 pairwise species combinations, 14 revealed significant differences (Table 3). As already shown in the overall comparison of 34 species, differences were mainly based on the variability of 1,4-dimethoxybenzene and *trans*-β-ocimene. These two compounds explained 84% of the observed total variability among this subset of eight

Table 3 Test statistics (R) of pairwise species comparison (ANOSIM)

	S. caprea	*S. cinerea*	*S. myrsinifolia*	*S. fragilis*	*S. viminalis*	*S. repens*	*S. triandra*	*S. bicolor*
S. caprea								
S. cinerea	0.123							
S. myrsinifolia	**1**[a]	**0.728**						
S. fragilis	**0.976**	0.605	**0.872**					
S. viminalis	**0.969**	0.483	1	0.619				
S. repens	**0.743**	0.472	0.316	0.441	0.594			
S. triandra	**0.9**	0.214	**0.817**	0.573	**0.786**	–0.056		
S. bicolor	**0.728**	**0.709**	0.644	**0.745**	**0.781**	0.017	0.106	

[a] Bold values indicate significant differences between two species. All species are likely to grow sympatrically, except for the subalpine *S. bicolor*.

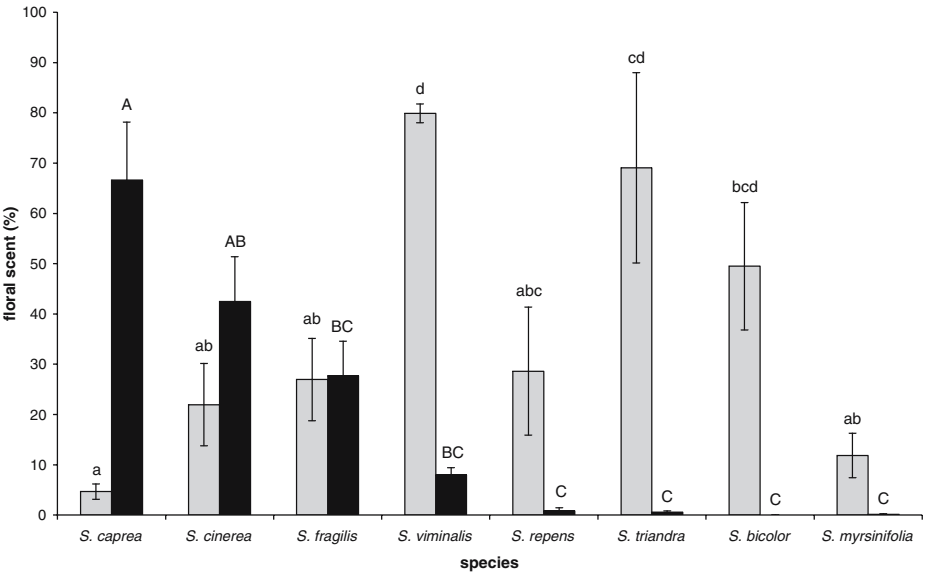


Fig. 3 Relative amount of 1,4-dimethoxybenzene (black) and *trans*-β-ocimene (*grey*) of the total floral scent in the most extensively sampled *Salix* species (ANOVA with Tukey–HSD test as post hoc procedure: $F_{df=7;55}=17.0$; $P<0.001$). *Different small letters* indicate significant interspecific differences in the amount of *trans*-β-ocimene and, *different capital letters* indicate significant interspecific differences in the amount of 1,4-dimethoxybenzene

species. For example, *S. caprea* and *S. cinerea* emitted much higher amounts of 1,4-dimethoxybenzene compared to *trans*-β-ocimene. Others, e.g., *S. viminalis*, *S. triandra*, and *S. bicolour*, were dominated by *trans*-β-ocimene (Fig. 3). *S. fragilis* was characterized by equally high amounts of 1,4-dimethoxybenzene and *trans*-β-ocimene. In *S. repens* and *S. myrsinifolia*, there was no clear predominance of a single compound; *trans*-β-ocimene content was below 30% and 1,4-dimethoxybenzene occurred only in traces.

Intraspecific Variation The variability within species in at least three out of eight species can be explained by sex differences (ANOSIM: $R=0.405$; $P<0.001$; Fig. 2). In *S. fragilis* ($N=6$), males emitted higher relative amounts of *trans*-β-ocimene and 1,4-dimethoxybenzene, whereas females contained more D-limonene and D-verbenone. In *S. myrsinifolia* ($N=6$), males emitted higher amounts of α-and β-pinene, while females emitted higher amounts of *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, and *trans*-β-ocimene. In *S. triandra* ($N=5$), females emitted higher amount of *trans*-β-ocimene while males released more β-pinene, *cis*-3-hexenyl acetate, D-limonene, and linalool. In the remaining five species, intraspecific variation as shown in Fig. 2 cannot be explained by sex differences.

Discussion

Floral scent emission as found in *Salix* is typical for entomogamous species. Indeed, willows are visited during the day and also at night by many insect species, e.g., bees, flies, beetles, butterflies, and moths (Vroege and Stelleman, 1990; Tollsten and Knudsen, 1992;

Tollsten and Sottocoornola, 2001; Karrenberg et al., 2002), and floral scents are probably important attractants.

Many floral scent compounds identified in *Salix* species are typical floral odors (compare e.g., Knudsen et al., 2006) and several are effective attractants for different insects (see below). This supports that in most willow species, flower-visiting insects are probably attracted by floral scents and thereby promote pollination. However, some of the detected components have been described as typical green leaf volatiles (e.g., *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, and (*E*)-4,8-dimethyl-1,3,7-nonatrien; Andersen et al., 1988; Whitman and Eller, 1990; Pare and Tumlinson, 1999; Ruther, 2000; Tholl et al., 2006) or have been found in leaves and/or other vegetative parts of different *Salix* species (Füssel et al., unpublished data). Green leave volatiles are likely to be produced in vegetative parts of the inflorescences, e.g., rhachis, flower bracts, and especially the leaves at the base of the catkins, which are, depending on species, more or less developed during flowering. Nevertheless, pollinators may detect, especially from long distances, the odor emitted from a whole plant and can use it as an olfactory cue to find their host plant and its flowers (e.g., Grison-Pigé et al., 2002). Therefore, in terms of pollinator attraction, we did not discriminate among compounds emitted by vegetative parts and by flowers, and refer to both as flower scent.

Compared with Tollsten and Knudsen (1992), who investigated floral scents in three species that we studied also—i.e., *Salix caprea*, *S. cinerea*, and *S. repens*, the results are similar considering both qualitative and quantitative aspects of scent composition. Tollsten and Knudsen (1992) identified 31 compounds, while we detected 34. Both studies found that *S. caprea* and *S. cinerea* are dominated by 1,4-dimethoxybenzene, while *S. repens* is dominated by a set of isoprenoids. However, despite these similarities, small differences exist. Tollsten and Knudsen (1992) identified four components (myrcene, 1,8-cineole, an oxygenated monoterpene, 2-phenyl ethyl methylether), which we did not detect. We identified α- and β-phellandrene, D-verbenone, and indole, which were not reported by Tollsten and Knudsen (1992). Surprisingly, we found one compound, benzaldehyde, in 31 of 34 species including *S. caprea*, *S. cinerea*, and *S. repens*, that was not reported by Tollsten and Knudsen (1992). However, all these differences concern only minor components of the total floral scent bouquet of a species. They might have been found in one study but not in the other because they fall below detection limits in some samples. In particular, benzaldehyde may be an artifact built by heating Tenax TA during desorbtion of the volatiles in the injector of the gas chromatograph (Peters et al., 1994). Several other factors also may be responsible for differences. First, different methods were used in the two studies (different adsorbents, thermodesorption vs. extraction of volatiles from filter using solvent). Second, Tollsten and Knudsen (1992) collected scent from cut twigs that were placed into water, whereas we collected scent from flowering twigs *in situ*. Some studies have shown differences in scent composition of flowers still attached to the living plant compared to that of flowers from cropped twigs (Mookherjee et al., 1990). Finally, geographic variability in floral scent of the three species could explain observed differences. Tollsten and Knudsen (1992) analyzed Swedish specimens growing wild while we analyzed specimens growing in southern Germany. Studies of other plant species document that that specimens originating from different populations emit differing relative amounts of compounds or even different compounds (e.g., Knudsen, 2002; Dötterl et al., 2005b; Svensson et al., 2005; Raguso et al., 2006).

Indeed, differing geographic origin might explain the intraspecific variability found in two of eight extensively sampled *Salix* species. The two species with samples originating from four or more origins (*S. repens* and *S. bicolor*), show a similarly high variability, while

the other six extensively sampled species originating from one or two origins are less variable. We cannot confirm Tollsten and Knudsen's (1992) finding that sex differences are responsible for the highly variable pattern of compounds in *S. repens* because plants studied in the Ecological–Botanical Garden of Bayreuth originated from five different geographic regions, thus masking possible sex differences within populations. *S. repens* is also morphologically a variable taxon, and floral scent might follow the same trend.

Relatively little is known about sex-specificity of floral scent in dioecious species. At least small differences in profiles between sexes have been found in some studies (e.g., Tollsten and Knudsen, 1992; Ashman et al., 2005). Ashman et al. (2005) reported that pollinators discriminated in gynodioecious *Fragaria* the scent of hermaphrodite flowers over those of females primarily because of the scent of hermaphrodite anthers. The anthers emitted high amounts of 2-phenylethanol, a benzoid compound found only in small amounts in female flowers. A comparison of the floral scent profiles of the three *Salix* species having significant sex differences with pollen scent profiles showed only differences in relative amounts, but no qualitative differences (U. Füssel et al., unpublished data) indicating that observed differences in scent between sexes cannot be explained by the emission of additional pollen-specific compounds in male flowers. Compared to male plants, which offer both pollen and nectar, female plants offer only nectar, and are, therefore, less attractive to insects collecting or eating pollen, such as beetles or bees (see also Ashman et al., 2005). Nevertheless, potential pollinators must be attracted to both male and female flowers for pollination to occur. The general view is that signals of male and female flowers have to correspond to promote successful pollen transfer. Consequently, it is usually assumed that flower visitors use similar cues to obtain rewards from female and male flowers (see reviews in Chittka and Thompson, 2001). This implies that insects seek similar rewards from both sexes. If this is not the case, e.g., when pollen is the desired reward (or females produce less or no nectar), nonrewarding female flowers are apparently pollinated by deceit due to their resemblance to rewarding male flowers (Baker, 1976). Contradictory to this intersexual mimicry hypothesis, the overall resemblance of male and female flowers in willows, especially with respect to visual cues, is low, and selection for resemblance of olfactory but not of visual cues seems to be unlikely, unless we assume that visual cues are of negligible importance for pollinator attraction. However, while nocturnal moths are probably more dependent on olfactory cues than day-active flower visitors, the situation might be completely different in day-active bees. For example, Galizia et al. (2004) found no evidence of olfactory mimicry in a (nectar) food-deceptive flower mimicry system. Their results indicate that in a bee-visited orchid evolutionary pressure acts on visual, but not olfactory traits toward a higher similarity to its model. Odor mismatch did not prevent bees from landing on flowers that had the expected visual display.

An alternative hypothesis, the specialized female reward hypothesis offered by Hemborg and Bond (2005) challenges the idea that pollinators search for the same reward in all conspecific flowers. According to Hemborg and Bond, males and females both offer essential, but different, components to the pollinators, and these sex-specific rewards may be advertised by sexually dimorphic floral signals. Kay (1985) and Elmqvist et al. (1988) found that female *Salix* flowers produce more nectar than male flowers, and Katoh et al. (1985) reported that females tend to have hexose dominated nectar in contrast to sucrose dominated nectar in males. Our own observations (Füssel et al., unpublished data) support these findings. Moreover, in case of the pollen specific bee *Andrena vaga*, it is known that females mainly collect pollen (and nectar) on some days, and on other days they feed on and/or collect only nectar from *Salix* (see Bischoff et al., 2003). Bees could use differences in scent of sex morphs to distinguish sexes, and to visit primarily/exclusively females when

focusing on nectar, and males when focusing on pollen. Additionally, nectar-seeking flower visitors in general could choose their preferred nectar source from the two sexes, thus fulfilling their actual needs. However, studies of specialization of female nectar rewards in entomogamous willows are scarce, and bioassays that prove if and how flower visitors differentiate between male and female attractants are lacking. Furthermore, pollen carry over only during occasional behavioral switches might be insufficient to ensure pollination. Therefore, from the plant point of view, similarity between the sexes is probably desirable to prevent pollinators from discriminating between male and female plants and to promote frequent cross-pollination.

It is interesting to note that *S. repens*, which emits a weak (Tollsten and Knudsen, 1992) and highly variable (Tollsten and Knudsen, 1992; present study) scent in comparison to other *Salix* species, seems to be primarily wind-pollinated (Vroege and Stelleman, 1990). Thus, the selective pressure to display a consistent pollinator-type specific floral scent profile across populations, sexes, and individuals might be lower, compared to species that are strongly dependent on insect pollination. Only in predominantly entomophilous species can distinct species-specific scent profiles promote flower constancy thus avoiding pollen waste, and functioning as reproductive isolation barriers between species.

On one hand, plants from quite different systematic positions have evolved the same pollination system involving the emission of similar floral scent spectra (e.g., Knudsen and Tollsten, 1993; Andersson et al., 2002). On the other, closely related plant species may have evolved quite different floral scent profiles that function as reproductive isolation barriers (e.g., Mant et al., 2005). If floral scent is to act as a willow specific attractant, pollinators must be able to perceive willow-specific floral scent compounds. If floral scent is to function as a reproductive isolation mechanism among willows, a second assumption has to be fulfilled: species must emit species-specific scents that pollinators may use to discriminate.

Dötterl et al. (2005a) demonstrated that floral scent compounds of *Salix* can act as cues for the attraction of oligolectic bees that collect pollen for their larvae exclusively on willows. *A. vaga* Pz. (Andrenidae) responded in GC-EAD tests to 1,4-dimethoxybenzene and other willow compounds; 1,4-dimethoxybenzene, in particular, attracted female bees in bioassays. Also many other *Salix*-visiting bee taxa respond strongly in GC-EAD tests to 1,4-dimethoxybenzene (Füssel et al., personal observation). It might be that 1,4-dimethoxybenzene, one of the two most common floral volatiles in willows, is the specific cue that provokes the flower constancy in these bees. It would be interesting to test whether *A. vaga* prefers 1,4-dimethoxybenzene dominated willow species over *trans*-β-ocimene dominated species.

Several other compounds found in this study are known to be attractive also to different insects or are at least active in electrophysiological studies. Beetles can be attracted by 1,4-dimethoxybenzene (Ventura et al., 2000); phenylacetaldehyde is attractive to flies (Howse, 2003), butterflies (Honda et al., 1998; Omura et al., 1999; Andersson, 2003), and moths (Haynes et al., 1991; Cunningham et al., 2004); and lilac aldehydes are known to be detected by butterflies (Andersson, 2003) and to be highly attractive to noctuid moth species (Dötterl et al., 2006). Besides *Andrena vaga* (Dötterl et al., 2005a), many other *Salix*-visiting bee taxa respond strongly in GC-EAD tests to 1,4-dimethoxybenzene and other typical willow volatiles (Füssel et al., personal observation.). Moreover, different willow-visiting moth species (e.g., *Orthosia* spp.) respond strongly in GC-EAD tests to several of the volatile compounds found in *Salix* (such as benzyl nitrile, lilac aldehyde A, and 4-oxoisopherone). It can be hypothesized that, besides 1,4-dimethoxybenzene and *trans*-β-ocimene that mainly distinguish species, several other flower scent compounds that are frequently found in *Salix* species may be effective specific attractants of potential pollinators. It is likely that they also

provide species-specific signals that serve as reproductive isolation barriers. In a subset of eight extensively sampled species, we showed a characteristic floral scent composition for six species, and half of the pairwise species comparisons confirmed significant differences. The observed differences are mainly based on the variability of only two compounds—1,4-dimethoxybenzene and *trans*-β-ocimene that together explain 84% of variability among these eight species. Of special interest are compatible species that grow sympatrically in the same habitats. According to Lautenschlager-Fleury and Lautenschlager-Fleury (1994) and Rothmaler (2002), this is the case for seven of the eight; only the subalpine *S. bicolor* is unlikely to cooccur with any of the other species. Nevertheless, even though these willows show significantly different scent profiles, natural hybrids are observed between most (see Rothmaler, 2002); even the subalpine *S. bicolor* is known to hybridize naturally with *S. caprea* and *S. myrsinifolia*.

Our results confirm those of Tollsten and Knudsen (1992) who found no significant differences between *S. caprea* and *S. cinerea*. However, their hypothesis that this high similarity in scent is responsible for frequently occurring hybridization between these species is not supported by our data on six other extensively sampled species. It seems that neither different scent profiles hamper hybridization, nor that similar scent profiles imply a higher risk of hybridization. However, because quantitative data on hybridization events between species pairs are lacking, this hypothesis needs further investigation.

Acknowledgements Parts of the study were supported by the German Research Foundation (Research Training Group 678). The authors thank F. Beyer and I. Schäffler for floral scent collection, and A. Lieflaender, A. Reuter, M. Suckling, T. Witt, and two anonymous reviewers for valuable comments on the manuscript.

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J Chem Ecol (2007) 33:767–779
DOI 10.1007/s10886-007-9264-7

A Comparison of Semiochemically Mediated Interactions Involving Specialist and Generalist *Brassica*-feeding Aphids and the Braconid Parasitoid *Diaeretiella rapae*

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Received: 9 October 2006 / Accepted: 7 February 2007 /
Published online: 28 February 2007
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Abstract *Diaeretiella rapae*, a parasitoid that predominately specializes in the parasitism of *Brassica*-feeding aphids, attacks *Lipaphis erysimi*, a specialist feeding aphid of the Brassicaceae and other families in the Capparales, at a greater rate than the generalist-feeding aphid, *Myzus persicae*. In this study, we investigated the orientation behavior of *D. rapae* to the volatile chemicals produced when these two aphid species feed on turnip (*Brassica rapa* var *rapifera*). We showed no significant preference orientation behavior to either aphid/turnip complex over the other. Isothiocyanates are among the compounds emitted by plants of the Brassicaceae in response to insect feeding damage, including by aphids. We assessed parasitoid orientation behavior in response to laboratory-formulated isothiocyanates. We tested two formulations and discovered significant orientation toward 3-butenyl isothiocyanate. We also assessed plant and aphid glucosinolate content, and showed large levels of glucosinolate concentration in *L. erysimi*, whereas there was little change in plant content in response to aphid feeding. Our results suggest that during the process of host location, similar cues may be utilized for locating *L. erysimi* and *M. persicae*, whereas the acceptance of hosts and their suitability may involve aspects of nonvolatile aphid chemistry.

Keywords Glucosinolate · Isothiocyanate · *Lipaphis erysimi* · *Myzus persicae* · *Diaeretiella rapae* · Volatiles · Orientation · Behavior

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Introduction

For insect parasitoids to reproduce they need to find suitable hosts. They must be efficient at using cues that indicate the presence of their hosts, and upon finding a potential host they must accurately assess its suitability. Vinson (1976, 1985) described successful host foraging behavior by insect parasitoids as a succession of five steps; host-habitat location, host location, host acceptance, host suitability, and host regulation. During the first three stages, collectively termed host selection, a range of volatile chemical species provide olfactory cues. These chemicals may be derived from host plants, hosts, or a complex of the two, which have been shown to be more attractive to foraging parasitoids or predators than the individual components of the system (see reviews by Dicke et al., 1990; Vet and Dicke, 1992; Vinson, 1999; and specifically for aphids Guerrieri et al., 1993, 1997; Reed et al., 1995; Du et al., 1996, 1997, 1998; Micha et al., 2000; Havill and Raffa, 2000).

Diaeretiella rapae is a braconid that predominately specializes in the parasitism of aphids feeding on the order Capparales, especially the Brassicaceae. *Diaeretiella rapae* attacks *Lipaphis erysimi*, a specialist feeder on the Capparales, at a greater rate than the generalist-feeding aphid *Myzus persicae* (Blande et al., 2004). This behavior is linked to cues detected by parasitoids upon emergence from their mummy cases, possibly glucosinolates that are characteristic, but not limited to the Brassicaceae. *D. rapae* would gain an advantage if they had the ability to orient toward the odor of a *L. erysimi*/brassica complex over that of a *M. persicae*/brassica complex.

In Brassicaceous plants, cell damage facilitates the hydrolysis of glucosinolates by myrosinase enzymes to produce a range of volatile catabolites that include nitriles and isothiocyanates (reviewed by Bones and Rossiter, 2006). After myrosinase catalyzed hydrolysis, the presence of epithiospecifier protein (ESP) generates the production of nitriles and epithionitriles from alkyl and alkenyl glucosinolates, respectively, whereas isothiocyanates are produced from any glucosinolate when ESP is absent (Kliebenstein et al., 2005). Isothiocyanates are thought to be important plant defense compounds that may provide parasitoids with cues that can be utilized in the host foraging process (Reed et al., 1995; Vaughn et al., 1996; Bradburne and Mithen, 2000). Preliminary analysis of the isothiocyanates produced by turnip indicated that infestation by both *L. erysimi* and *M. persicae* is responsible for elevating emissions of 3-butenyl isothiocyanate and 4-pentenyl isothiocyanate (Blande, 2004).

We hypothesized that differences in the strategies of the two aphid species would result in differences in the volatile profiles emitted by the plants, and that foraging *D. rapae* would preferentially orient toward the *L. erysimi*/turnip complex over the *M. persicae*/turnip complex. To explore the foraging behavior of *D. rapae* we utilized a Y-tube olfactometer and conducted a series of choice tests with naïve parasitoids and individuals that had previously experienced a plant/aphid complex. We also looked at the responses of aphid parasitoids to pure laboratory formulations of isothiocyanates.

In addition to the orientation behavior of *D. rapae*, we investigated the nonvolatile glucosinolate chemistry of the plants and aphids in the system. Previous analyses have been conducted on turnip, *Brassica rapa* var *rapifera* (Carlson et al., 1981, 1987). Other studies have documented evidence of glucosinolate accumulation in several insect species that specialize at feeding on the Brassicaceae (Aplin et al., 1975; Weber et al., 1986; Müller et al., 2001; Bridges et al., 2002).

The brassica-specializing aphids *Brevicoryne brassicae* and *L. erysimi* are further adapted to feeding on the Brassicaceae by producing their own myrosinase (MacGibbon and Allison, 1970; MacGibbon and Beuzenberg, 1978; Jones et al., 2001), which can

hydrolyze sequestered glucosinolates that act synergistically with (*E*)-β-farnesene in an alarm system (Dawson et al., 1987; Jones et al., 2001; Bridges et al., 2002; Husebye et al., 2005). This myrosinase is localized to the muscle tissue of the head and thorax in *B. brassicae*, and is also present in *L. erysimi*. It has been categorized down to its X-ray structure (Husebye et al., 2005). We hypothesized that like the better categorized specialist *B. brassicae* (Bridges et al., 2002), *L. erysimi* would accumulate glucosinolates that can be used in defense whereas the generalist-feeding *M. persicae* would pass glucosinolates through the gut (Weber et al., 1986; Cole, 1996). We also hypothesized that the glucosinolate content of the plants could be differentially affected by aphid feeding because of variable plant defense responses. We addressed this by analysis of desulfoglucosinolates in the plant and aphid tissue.

Methods and Materials

Parasitoid Rearing and Standardization of Insects *Diaeretiella rapae* (McIntosh) from stocks at Rothamsted Research reared on *Myzus persicae* on Chinese cabbage were established as separate cultures on *M. persicae* and *L. erysimi*, both on turnip. The original stocks were started in 1990, and were supplemented in 1995 with 30 females and 20 males collected from oilseed rape.

Some experiments utilized "naïve" female parasitoids that had no adult experience of aphids or plant material besides that received from the surface of the mummy case during and after emergence (Poppy et al., 1997). All experimental parasitoids were removed from turnip plants as mummified aphids with watchmaker forceps and stored in darkened plastic containers attached to glass jars. Newly emerged adult parasitoids congregated in the jars as they displayed positive phototaxis. At 24-hr intervals, the glass jars containing parasitoids were removed and screwed onto a lid supporting a cotton wool ball soaked with 25% honey solution. At the same time, a clean jar was affixed to the plastic container. Parasitoids collected in the glass jars were left for 3 d so that all experimental insects were standardized mated females, with no adult experience of aphids or plants (Du et al., 1996, 1997, 1998). Before experiments, parasitoids were sexed and separated into glass vials with honey solution provided on tissue paper twists. All experiments were conducted at 21°C under a laminar light source, with a light intensity of 115 μmol s^{-1} m^{-2} measured at bench level.

Preparation of Plants Turnip plants, *B. rapa* var *rapifera* cv Tokyo Cross, were cultivated in glasshouses at Rothamsted Research, Harpenden, U.K. Before they were used as odor cues, plants aged 4 wk were removed from glasshouses and transferred to controlled environment rooms with a photoperiod of 16 light/8 dark; plants were infested with *L. erysimi* (Kaltenbach), *M. persicae* (Sulzer), or left uninfested. Individual plants were infested with approximately 500 aphids of mixed instars. All plants were left for 5 d before use as odor sources in Y-tube bioassays.

Y-tube Experiments A Y-tube olfactometer, described in detail in Du et al. (1996, 1997), was used to compare the orientation behavior of *D. rapae* to different odors. Air was pumped by a Capex L2× pump (Charles Austen Pumps Ltd., Byfleet, Surrey, UK) through an activated charcoal filter, and was subsequently limited by a flowmeter to 800 ml/min, then split by a brass T-junction fitting (Swagelock, OH, USA), and passed into two airtight glass chambers (Soham Scientific) that contained the test odor sources. Flow meters were used to adjust the airflow from the chambers to 400 ml/min, which was then channeled into

the two short ends of a glass Y-tube olfactometer (diam. 1 cm) (Soham Scientific). Teflon tubing (diam 1 mm) was used to channel air through the system. This tubing was affixed to the ends of the Y-tube with rubber bungs. Individual female parasitoids in glass vials were released into the Y-tube by placing the vials next to the opening of the tube. Parasitoid behavior was observed, and the arms of the olfactometer selected by the parasitoids were recorded. In all experiments, an odor selection was recorded when a parasitoid advanced further than halfway along a Y-tube arm, touched the rubber bung at the end of the arm, and remained in that half of the Y-tube arm for 15 sec. Between bioassay sets, the glass vessels and Y-tubes were washed in soapy water and rinsed with bulk acetone and glass distilled water. Charcoal filters were baked overnight at 180°C with a constant nitrogen stream passed through them.

Experiment 1: Response of Naïve D. rapae to Plants and Plant/Host Complexes Naïve female *D. rapae*, reared on both *L. erysimi* and *M. persicae*, were used in these bioassays to compare responses to odors of different plant/host complexes. Plant/host complexes (turnip/*L. erysimi* and turnip/*M. persicae*) were used in bioassays vs. blanks, which consisted of distilled water on filter paper (Whatman no. 1) to reduce differences in humidity of the air entering each arm of the Y-tube and clean undamaged plants. The plant/host complexes were also bioassayed opposite each other.

Experiment 2: Effect of Adult Associative Learning Experience on Parasitoid Response to Odors Diaeretiella rapae reared on both *L. erysimi* and *M. persicae* were used in these bioassays. Parasitoids reared and treated in the same way as naïve parasitoids were provided with a postemergence experience. This was implemented by releasing parasitoids into a cage that contained a turnip plant infested with 500 aphids 5 d previously, exactly the same treatment given to plants used as odor sources in the subsequent bioassays. Each experience period lasted for approximately 3 min, and all parasitoids utilized in bioassays were observed to have made an ovipositional attack. After 3 min, the parasitoids were collected by using an electric pooter, and then separated into glass vials containing honey solution on tissue paper twists and left for approximately 2–3 hr before they were used in bioassays (Du et al., 1996, 1997). The plant/host complexes used in the bioassays were *L. erysimi* on turnip and *M. persicae* on turnip. These complexes were bioassayed opposite clean turnips and each other.

Experiment 3: Responses of D. rapae to Synthetic Isothiocyanates 3-Butenyl isothiocyanate and 4-pentenyl isothiocyanate were synthesized at Rothamsted Research (Dawson et al., 1993). The isothiocyanates were of greater than 97% purity and made to a concentration of 1 mg/ml in redistilled ether. Isothiocyanate solution was applied to Whatman no. 1 filter paper in 50 µl volumes with a Gilson pipette. Isothiocyanates were bioassayed opposite blanks, which consisted of 50 µl of redistilled ether on filter paper, and in opposition with each other. Naïve female *D. rapae* reared on *L. erysimi* and *M. persicae* were utilized in the bioassays.

Statistical Analysis Data collected from all behavioral bioassays were analyzed by using chi-square tests, which were adjusted for data containing just two categories by using Yates' correction. Parasitoids not making a selection were removed from the data set used in the chi-squared analysis. At least 50 parasitoids were tested for each bioassay combination.

Plant Preparation for Glucosinolate Analysis Turnip plants aged 30 d were infested with 250 aphids of mixed instars, three plants with *M. persicae*, and three with *L. erysimi*. Three

plants were not infested. All plants were kept at 18°C for 5 d before preparation for glucosinolate analysis. Four leaves were incised from the whorl within the outer two leaves of the now 35-d-old turnip plants. Aphids were removed by washing with distilled water, and leaves from the noninfested control plants were also washed. Leaves were shaken dry and wrapped in tin foil. Leaves from each plant were separately wrapped and placed in a freeze drier for 72 hr before being ground into a fine powder with a pestle and mortar and packaged into plastic tubes (Bibby Sterilin Ltd., Staffordshire, UK).

Aphid Preparation for Glucosinolate Analysis Aphids were removed from two plants heavily infested with *M. persicae* and two heavily infested with *L. erysimi*. A fine paintbrush was used to transfer the aphids from the plants into plastic tubes. Thousands of aphids were required to provide sufficient material for analysis. Tubes were sealed with gauze, which was fastened to the top of the tubes with elastic bands, and then freeze dried for 48 hr. Aphids were ground into a fine powder with a pestle and mortar and packaged into plastic tubes.

The glucosinolate content of the prepared plant and aphid samples was qualitatively and quantitatively analyzed by J. Marquez (School of Biosciences, University of Nottingham). The procedure used involved processing the material to produce desulfoglucosinolates, which were then separated and quantified by using high-performance liquid chromatography (HPLC) with sinigrin as an internal standard; the methods are described in detail in Magrath et al., (1993).

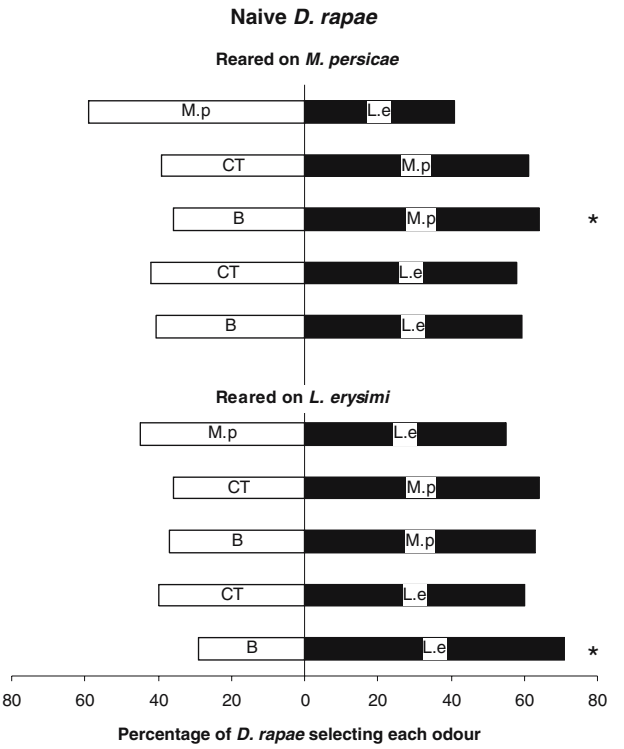


Fig. 1 Response of naïve female *D. rapae* in a Y-tube olfactometer when offered *M. persicae*-infested turnip (M.p), *L. erysimi*-infested turnip (L.e), clean turnip (CT), and distilled water blanks (B). *Diaeretiella rapae* were reared on either *M. persicae* or *L. erysimi*, as indicated. Asterisk indicates a significant difference ($P<0.05$; $N\geq50$)

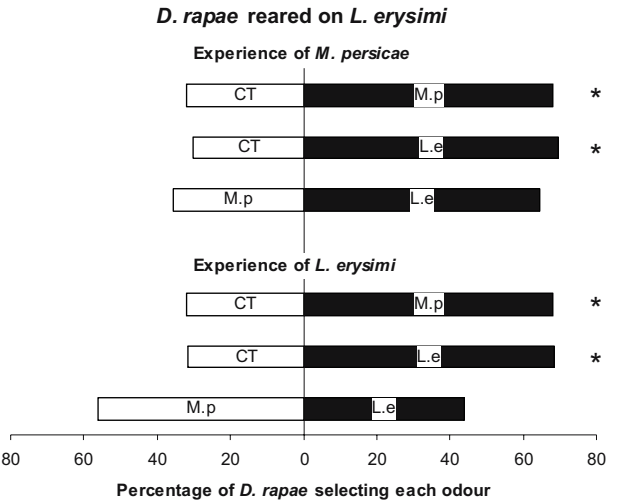


Fig. 2 Response of experienced female *D. rapae* in a Y-tube olfactometer when offered *M. persicae*-infested turnip (M.p), *L. erysimi*-infested turnip (L.e), or clean turnip (CT). *Diaeretiella rapae* were reared on *L. erysimi* and exposed to either *M. persicae*- or *L. erysimi*-infested turnip to provide an experience. Asterisk indicates a significant difference ($P<0.05$; $N\geq50$)

Results

Responses of Naïve D. rapae to Turnip Plants and Turnip/Aphid Complexes Naïve *D. rapae* reared on *M. persicae* made significantly more selections of a Y-tube arm leading toward a *M. persicae*-infested turnip plant than a blank ($P<0.05$), but other bioassays revealed no significant differences in the response to the odors presented (Fig. 1). *Diaeretiella rapae* reared on *L. erysimi* selected *L. erysimi*-infested turnip significantly more often than blanks ($P<0.05$). There were no other significant odor selections (Fig. 1).

Effect of Postemergence Experience on Parasitoid Response to Odors Diaeretiella rapae parasitoids reared on both *L. erysimi* and *M. persicae* were given postemergence experience of either the host/plant complex or the alternative host/plant complex. The host/plant complex is exactly the same system upon which the parasitoids were reared. Experience of

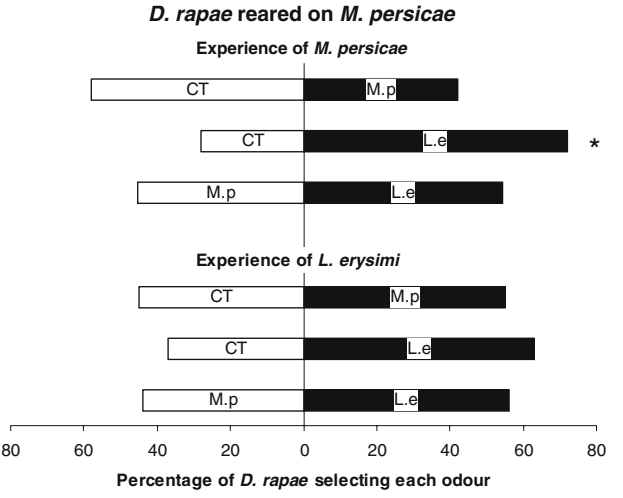


Fig. 3 Response of experienced female *D. rapae* in a Y-tube olfactometer when offered *M. persicae*-infested turnip (M.p), *L. erysimi*-infested turnip (L.e), or clean turnip (CT). *Diaeretiella rapae* were reared on *M. persicae* and exposed to either *M. persicae*- or *L. erysimi*-infested turnip to provide an experience. Asterisk indicates a significant difference ($P<0.05$; $N\geq50$)

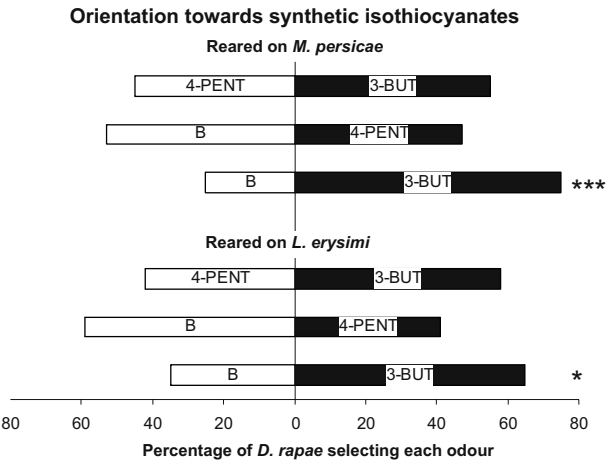


Fig. 4 Response of naïve female *D. rapae* in a Y-tube olfactometer when offered 4-pentenyl isothiocyanate, 3-butenyl isothiocyanate, or redistilled ether blanks (B). *Diaeretiella rapae* were reared on either *M. persicae* or *L. erysimi*, as indicated. Single asterisk indicates a significant difference ($P<0.05$); triple asterisks indicate a significant difference ($P<0.001$; $N\geq50$)

this system is referred to as a "home" experience. An experience of the alternative host/plant complex that is the same plant that the parasitoids were reared on but an alternative aphid species is referred to as an "away" experience. *Diaeretiella rapae* reared on *L. erysimi* selected the odor of both home and away complexes on a significantly greater number of occasions than that of clean plants ($P<0.05$), irrespective of the complex experienced (Fig. 2). *Diaeretiella rapae* reared on *M. persicae* given a home experience showed a significant attraction to the away complex ($P<0.05$), but there was no other significant attraction displayed (Fig. 3).

D. rapae Response to Synthetic Isothiocyanates Naïve female *D. rapae* significantly oriented toward 3-butenyl isothiocyanate when bioassayed opposite a blank consisting of an equal volume of ether, irrespective of whether they were reared on *M. persicae* ($P<0.001$) or *L. erysimi* ($P<0.05$). There was no significant attraction to 4-pentenyl isothiocyanate. When the two isothiocyanates were in competition with each other, there was no significant attraction to either (Fig. 4).

Table 1 Glucosinolate composition of turnip plants infested with *M. persicae* or *L. erysimi*, and the glucosinolate composition of the aphids

Glucosinolates	Le Turnip (%)	Mp Turnip (%)	Cl Turnip (%)	Le aphids (%)	Mp aphids (%)
3-Butenylglucosinolate	7.8±1.5 (73.7)	6.3±1.83 (76.1)	6.4±1.29 (72.6)	4.02 (37.1)	0.29 (65.9)
Hydroxyindolylmethylglucosinolate	0±0 (0)	0±0 (0)	0±0 (0)	0.15 (1.39)	0 (0)
4-Pentenylglucosinolate	2.2±0.31 (20.9)	1.7±0.52 (20.5)	2.0±0.5 (22.7)	1.27 (11.7)	0.06 (13.6)
Indolylmethylglucosinolate	0.16±0.08 (1.5)	0.06±0.017 (0.72)	0.07±0.02 (0.79)	4.12 (38.1)	0.07 (15.9)
4-Methoxyindolylmethylglucosinolate	0.10±0.003 (0.95)	0.09±0.059 (1.1)	0.08±0.0089 (0.91)	0.41 (3.79)	0 (0)
2-Phenylethylglucosinolate	0.25±0.06 (2.4)	0.12±0.035 (1.45)	0.21±0.074 (2.38)	0.81 (7.5)	0 (0)
1-Methoxyindolylmethylglucosinolate	0.08±0.01 (0.76)	0.06±0.017 (0.72)	0.09±0.022 (1.02)	0.05 (0.46)	0.01 (2.27)
Total	10.5±2.08 (100)	8.3±2.44 (100)	8.8±1.85 (100)	10.82 (100)	0.44 (100)

The mean±SE quantities of the glucosinolates present in turnip leaf tissue ($N=3$) and aphids ($N=1$) are in micromoles per gram dry weight. Values in parentheses indicate the percentage of the total glucosinolate content represented by each individual component.

Le = *L. erysimi*, Mp = *M. persicae*, Cl = clean

Glucosinolate Analysis Analyses were conducted on clean turnip plants and plants that had been infested with *M. persicae* or *L. erysimi*. Six different glucosinolates were analyzed and were present in all the samples. These were the aliphatic glucosinolates, gluconapin (3-butenylglucosinolate) and glucobrassicanapin (4-pentenylglucosinolate), the simple aromatic gluconasturtin (2-phenylethylglucosinolate), and the indolylglucosinolates, glucobrassicin (indolylmethylglucosinolate), neoglucobrassicin (1-methoxyindolylmethylglucosinolate), and 4-methoxyglucobrassicin (4-methoxyindolylmethylglucosinolate).

The mean total glucosinolate content was greatest in *L. erysimi*-infested turnip, and lowest in *M. persicae* infested turnip, although the differences between the three treatments were small, and variation among plants receiving the same treatment was great. 3-Butenylglucosinolate was present in the greatest quantity, followed by 4-pentenylglucosinolate (Table 1).

Analysis of the glucosinolates present in the tissue of freeze-dried *L. erysimi* and *M. persicae* revealed large differences. *Lipaphis erysimi* contained large quantities of all the plant glucosinolates (3-butenylglucosinolate, 4-pentenylglucosinolate, 2-phenylethylglucosinolate, indolylmethylglucosinolate, 1-methoxyindolylmethylglucosinolate, and 4-methoxyindolylmethylglucosinolate) with the inclusion of hydroxyindolylmethylglucosinolate. *Myzus persicae* contained traces of 3-butenylglucosinolate, 4-pentenylglucosinolate, indolylmethylglucosinolate, and 1-methoxyindolylmethylglucosinolate, but 4-methoxyindolylmethylglucosinolate, 2-phenylethylglucosinolate, and hydroxyindolylmethylglucosinolate were absent, or in traces too small for detection by HPLC (Table 1).

Discussion

Naïve *D. rapae* tended to orient toward both home and away aphid/turnip complexes in preference to the clean turnip plants or blanks. This was irrespective of the original host aphid species. These results complement previous studies that showed orientation of aphid parasitoids to plant/aphid complexes (Read et al., 1970; Reed et al., 1995; Vaughn et al., 1996; Powell et al., 1998). However, the only statistically significant orientations were toward the home complex opposed to a blank, and this was the same irrespective of the original host. Parasitoids did not orient toward one complex over the other.

The environment in which parasitoids develop has been shown to influence adult response to volatile chemicals (Sheehan and Shelton, 1989; van Emden et al., 2002), and emergence from mummy cases provides parasitoids with an experience that can prime behavioral responses (van Emden et al., 1996; Storeck et al., 2000). It was expected that naïve *D. rapae* would orient toward aphid-infested turnip plants in preference to clean plants. However, further experiences of aphid/plant complexes postemergence also influence behavior. Providing parasitoids with a positive experience that is associated with an odor can facilitate the effective use of volatile chemicals by parasitoids, or enhance their ability to respond to these odors, thus increasing foraging efficiency (Godfray and Waage, 1988; Vet and Groenewold, 1990; Micha et al., 2000; Mölck et al., 1999, 2000; Grasswitz and Paine, 1993).

The provision of *D. rapae* with an experience of an aphid/turnip complex appeared to heighten their ability to orient toward an aphid/turnip complex. This was particularly clear when the parasitoids had been reared on *L. erysimi*. However, there was still no difference in orientation to one complex in preference to the other. Our results conflicted with our hypothesis, which was based on attack rates (Blande, 2004), indicating that *D. rapae* would orient preferentially toward the odor of the *L. erysimi*/turnip complex. Previous studies by Du et al. (1996, 1997, 1998) on a broad bean (*Vicia faba*) system showed that *Aphidius ervi* parasitoids were able to orient toward the odor of their host/plant complex at a greater frequency

than to a nonhost/same plant complex. This indicates a specificity of the volatile signals emitted by plants in response to damage by different aphid species. However, it appears that *D. rapae*, irrespective of their original host or experience, are either incapable of distinguishing between the odors of the two complexes, or else do not have a preference for either of the odor options.

Earlier studies have suggested that feeding by different aphid species can elicit the emission of variable volatile blends because of differences in their saliva (Du et al., 1998; Guerrieri et al., 1999). Oral secretions from the Lepidoptera alter the volatile composition of corn seedlings, resulting in odors that parasitoids can distinguish as different from those produced by physical damage alone (Turlings et al., 1990). However, preliminary work in our system (Blande, 2004) indicated only quantitative differences between the volatile isothiocyanates produced by turnip infested by *M. persicae* and *L. erysimi*. It would be interesting to analyze the different gene transcriptional activity during feeding by these two species.

The volatile chemistries of the complexes did not appear differentially attractive when presented in a choice scenario. Previous work by Du et al. (1996), showed clear orientation toward a host/plant complex in preference to a nonhost/plant complex, but in this study, both aphid species used were suitable hosts for *D. rapae*. Foraging parasitoids would benefit from the ability to orient toward the volatiles emitted by either complex in the absence of the other, and it is possible that when volatiles from the complexes are present concurrently, other cues such as visual cues are also utilized.

Isothiocyanates have been suggested as important signaling chemicals in a few previous studies (Reed et al., 1995; Vaughn et al., 1996; Bradburne and Mithen, 2000), and we know that feeding by both *L. erysimi* and *M. persicae* induces the emission of 3-butenyl and 4-pentenyl isothiocyanates. In Y-tube bioassays, we showed that 3-butenyl isothiocyanate was significantly more attractive than the solvent alone, irrespective of the host aphid, but we did not observe orientation toward 4-pentenyl isothiocyanate. However, when the two isothiocyanates were used in opposing arms of the olfactometer, there was no significant attraction to 3-butenyl isothiocyanate. This might be because of the odor of 4-pentenyl isothiocyanate disrupting the *D. rapae* response to 3-butenyl isothiocyanate, without actually attracting the parasitoids. It appears that 3-butenyl isothiocyanate provides an important foraging cue for *D. rapae.*

The results of this investigation are interesting when considered alongside the attack behavior of *D. rapae* in which *L. erysimi* is attacked at a greater rate than *M. persicae*, irrespective of whether the original host was *L. erysimi* or *M. persicae* (Blande et al., 2004). This behavior suggests that *L. erysimi* may be a preferable host for *D. rapae*, and that *D. rapae* may orient toward this complex in preference to the *M. persicae* alternative. However, in this investigation, neither complex appeared more attractive than the other, although we have shown an attraction toward laboratory-formulated 3-butenyl isothiocyanate in Y-tube olfactometers. Our results suggest that during the process of host-habitat and host location the cues used to locate *L. erysimi* and *M. persicae* hosts are probably the same. Perhaps glucosinolate processing by aphids may influence host acceptance behavior.

Glucosinolates in turnip leaf tissue were not significantly altered in response to aphid feeding, whereas damage by the cabbage stem flea beetle (*Psylliodes chrysocephala* L.) has been shown to facilitate glucosinolate accumulation (Koritsas et al., 1989; Bartlet et al., 1999). As feeding by aphids causes minimal cellular damage (Miles, 1999), it is not surprising that plant responses may be different from those elicited by chewing herbivores. A recent study by De Vos et al. (2005) showed that three insect herbivore species with different feeding strategies and infection with a fungal or bacterial pathogen all result in different gene transcription activity in the model plant *Arabidopsis thaliana*. Surprisingly, feeding by *M. persicae* induced 10 times more gene transcriptional changes than feeding by the chewing herbivore *Pieris rapae* or the cell-content-feeding *Frankliniella occidentalis*. Although *M.*

persicae feeding elicits a large number of gene transcriptional changes in *A. thaliana*, the glucosinolate content of our plants did not increase in response to aphid feeding, perhaps suggesting that the cytochrome P450s encoded by the *CYP79* and *CYP83* gene families responsible for stages of glucosinolate biosynthesis (Grubb and Abel, 2006) were not upregulated. Possibly, plants under heavier aphid stresses and showing more physical damage symptoms will exhibit more differences in glucosinolate content, but the indications here are that the early stages of aphid infestation do not facilitate changes in plant glucosinolate content.

The results of the glucosinolate analysis of dried aphid material showed huge differences between the two species. *Myzus persicae*, as previously indicated, contained small quantities of glucosinolate, indicative of a strategy involving the passing of glucosinolates straight through the gut (Weber et al., 1986; Cole, 1996; Bridges et al., 2002), whereas *L. erysimi*, like *B. brassicae* (Bridges et al., 2002), sequestered these compounds. This supported our hypothesis and might be explained by previous work conducted that showed the presence of a myrosinase in the head and thorax regions of this aphid species (MacGibbon and Allison, 1970; MacGibbon and Beuzenberg, 1978; Bridges et al., 2002). Interestingly, the proportions of the different glucosinolate components in the aphid tissue did not match those of the plant leaf material. Plants leaves were dominated by 3-butenylglucosinolate and 4-pentenylglucosinolate, whereas although these glucosinolates were significant components in the aphid tissue, there was a large accumulation of indolylmethylglucosinolate, which was only a minor constituent of the total plant leaf glucosinolate. Furthermore, hydroxyindolylmethylglucosinolate was found only in *L. erysimi* tissue. The most likely explanation is that quantities were so low within the plants that they fell below the HPLC detection limits, but concentration in the aphid tissue allowed for detection in those samples.

The glucosinolates stored in the aphid tissue are likely to provide foraging parasitoids with cues that can be used to assess the quality of the prospective host for their eggs. Chemical compounds present in aphid cornicle secretions have been shown to stimulate oviposition attack responses in *Aphidius ervi* (Battaglia et al., 1993, 2000). As *D. rapae* attack *L. erysimi* at a greater rate than *M. persicae* (Blande et al., 2004), it is likely that glucosinolates provide important chemical signals. Sensory cells on the antennae and ovipositor (De Faris and Hopper, 1999; reviewed by Mackauer et al., 1996) are essential in the processes of host acceptance and suitability. However, in the process of host location, it is insect-induced volatile emissions that provide the signals to foraging parasitoids.

Acknowledgements We thank Julietta Marquez for her work on the glucosinolate analysis. J. Blande was supported by a Biotechnology and Biological Sciences Research Council of the United Kingdom (BBSRC) Quota studentship. Funding for this work was provided in part by the Department of the Environment, Food and Rural Affairs. Rothamsted Research receives grant-aided support from the BBSRC.

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J Chem Ecol (2007) 33:781–799
DOI 10.1007/s10886-007-9252-y

Evaluation of the Evolution of Increased Competitive Ability (EICA) Hypothesis: Loss of Defense Against Generalist but not Specialist Herbivores

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Received: 17 November 2006 / Accepted: 17 January 2007 /
Published online: 28 February 2007
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Abstract The evolution of increased competitive ability (EICA) hypothesis predicts that invasive plant species may escape their specialized natural enemies in their introduced range and subsequently evolve with a decrease in investment in anti-herbivore chemical defenses relative to native conspecifics. We compared the chemical profile of 10 populations of US native and 20 populations of European invasive *Solidago gigantea*. To test for differences in inducibility between native and invasive populations, we measured secondary chemistry in both damaged and undamaged plants. We also performed bioassays with three specialist and two generalist insect herbivores from four different feeding guilds. There was no evidence that invasive populations had reduced concentrations of sesquiterpenes, diterpenes, or short-chain hydrocarbons (SCH), although significant variation among populations was detected. Sesquiterpene and diterpene concentrations were not influenced by damage to the host plant, whereas SCH concentrations were decreased by damage for both native and invasive plants. Performance of the three specialist insects was not affected by the continental origin of the host plant. However, larval mass of the generalist caterpillar *Spodoptera exigua* was 37% lower on native plants compared to invasive plants. The other generalist insect, a xylem-tapping spittlebug that occurs on both continents, performed equally well on native and invasive plants. These results offer partial support for the defense predictions of the EICA hypothesis: the better growth of *Spodoptera* caterpillars on European plants shows that some defenses have been lost in the introduced range, even though our measures of secondary chemistry did not detect differences between continents. Our results show significant variation in chemical

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defenses and herbivore performance across populations on both continents and emphasize the need for analysis across a broad spatial scale and the use of multiple herbivores.

Keywords Diterpene · *Epiblema scudderiana* · Evolution of increased competitive ability · EICA · Hydrocarbons · Herbivory · Invasive · *Philaenus spumarius* · *Solidago gigantea* · *Spodoptera exigua* · *Trirhabda virgata* · *Uroleucon nigrotuberculatus*

Introduction

In the United States, nonnative and invasive plants cause damage and losses totaling more than US$34 billion per year (Pimentel et al., 2000) and threaten native ecosystems (Mack et al., 2000). Despite the economic importance of invasive plants and the attention devoted to understanding biological invasions, the mechanisms that allow plants to become invasive are poorly understood (D'Antonio and Kark, 2002). The enemy release (ER) hypothesis predicts that invading plants experience success in novel habitats due to a decrease in regulation by specialized herbivores and other natural enemies in the introduced range (Elton, 1958; Keane and Crawley, 2002). The loss of enemies in the introduced range can result in reduced herbivore and parasite loads on invasive species compared to native species (Wolfe, 2002; Mitchell and Power, 2003; Carpenter and Cappuccino, 2005; Cappuccino and Carpenter, 2005; Agrawal et al., 2005; Vilà et al., 2005), potentially allowing invasive species to outperform natives.

Blossey and Nötzold (1995) extended the ER hypothesis by reasoning that if natural enemy pressure on exotic plants is reduced in the introduced range, then plants should evolve and permanently decrease their investment in defenses and maximize their investment in growth and reproduction. The evolution of increased competitive ability (EICA) hypothesis (Blossey and Nötzold, 1995) predicts that in the absence of herbivores, costly chemical defenses should be reduced in invasive plants. In addition, this loss should increase host plant quality to herbivores. However, this prediction should only apply to specialist herbivores, as generalist herbivores are likely to be encountered in the introduced range (Müller-Schärer et al., 2004; Joshi and Vrieling, 2005). It is also possible that introduced plants could retain their ability to induce defenses against herbivores, as induced defenses should be less costly than constitutive defenses.

Some studies have supported the EICA hypothesis. Siemann and Rogers (2003a) showed that in common gardens, invasive Chinese tallow (*Sapium sebiferum*) were more prone to attack by natural enemies and that generalist herbivores preferred invasive over native genotypes (Siemann and Rogers, 2003b). In addition, invasive Chinese tallow tree genotypes planted in the invasive range had enhanced growth relative to native genotypes and had significantly lower levels of foliar tannins (Siemann and Rogers, 2003b). Other work has yielded mixed results. Invasive purple loosestrife (*Lythrum salicaria*) had significantly lower plant secondary metabolites than native plants; however, herbivore bioassays did not reveal differences between introduced and native populations (Willis et al., 1999). In multiple common gardens, invasive St. John's wort (*Hypericum perforatum*) were neither larger nor more fecund than native plants (Maron et al., 2004b), although they had lower pathogen resistance and exhibited lower levels of the chemical hypericide in invasive range gardens and lower pseudohypercide levels in native range gardens (Maron et al., 2004a). Reduced herbivore damage in European gardens of the invasive ragweed (*Ambrosia artemisifolia*) translated to increased plant growth, but not size or vigor (Genton

et al., 2005), and native genotypes were as damaged as invasive genotypes in multiple gardens, suggesting no evolutionary loss of defense against herbivores.

A few studies that test the EICA hypothesis have measured levels of defensive chemicals (see Hinz and Schwarzlaender, 2004 for review) and those that do typically only measure single compounds (but see Cipollini et al., 2005). In a comprehensive test of the chemical defense predictions of the EICA hypothesis, Cipollini et al. (2005) examined constitutive and induced levels of five chemical defense traits for native and introduced populations of *Alliaria petiolata*. Their results generally did not support the EICA hypothesis, as two compounds were found at higher constitutive levels in invasive plants, and there were few other continental differences. However, there was a tendency for plants from the introduced range to have lower constitutive levels of glucosinolates but higher inducibility. Previous studies that have used insect herbivores as bioassays for plant defenses generally use leaf-chewing insects and do not examine more than one or two insect herbivores (e.g., Willis et al., 1999; Siemann and Rogers, 2003a,b; Bossdorf et al., 2004; Stastny et al., 2005; Leger and Forister, 2005).

In the study reported here, we determined end-of-season secondary chemistry and its possible effects on multiple insect herbivores for *Solidago gigantea* Ait. (late goldenrod). *S. gigantea* is native to North America and was introduced into Europe as an ornamental (circa 1758), where it naturalized and began to spread by 1850 (Weber, 1998). Today, it is among the most aggressive invasive species in Europe (Weber and Schmid, 1998). *S. gigantea* is a clonal perennial that commonly spreads throughout an area via asexual rhizomes. There are several characteristics that make it particularly appropriate for a test of the EICA hypothesis. First, there is evidence that it has escaped its specialist natural enemies in Europe. In a comparison of 46 North American populations and 45 European populations of *S. gigantea*, Jakobs et al. (2004) found that most North American populations had signs of insect herbivory, and 70% were severely damaged, while none of the European populations was infested by insects (although two populations had limited damage). Similarly, Jobin et al. (1996) concluded that few of the insects feeding on the native European goldenrod, *Solidago virgaurea*, have switched to *Solidago altissima*, another North American invasive species, perhaps because *S. virgaurea* and the introduced *Solidago* spp. do not generally occur together in the same locations. Second, *S. gigantea* has been present in Europe for about 250 years, allowing time for genetic changes to occur. Third, *S. gigantea* plants growing in the field in Europe are larger and grow more densely than conspecifics in the US, consistent with predictions of the EICA hypothesis (Jakobs et al., 2004). Finally, when *Solidago canadensis* was treated with jasmonic acid, an elicitor of plant defensive compounds, the inflorescence biomass decreased, and flowering was delayed, indicating that in a closely related *Solidago* spp., the production of defensive compounds incur a fitness cost (van Kleunen et al., 2004).

Previous research with *S. gigantea* has shown some loss of defenses against natural enemies in European populations. When grown in a common garden in the US and exposed to naturally occurring insects and leaf pathogens for 2 years, European plants were consistently more susceptible to a rust fungus and a bacterial pathogen than US plants (Meyer et al., 2005). Results for insect herbivores were more equivocal, with European plants supporting a greater biomass of insect herbivores in the first year but not the second. A variety of insects colonized the garden over the 2-year study, with changing species composition and abundance between the 2 years, making this result hard to interpret (Meyer et al., 2005).

In the study reported here, we grew *S. gigantea* plants derived from multiple populations from the introduced range and the native range in common gardens. In accordance with EICA, we predicted that chemical defenses would be lower in the introduced range. We

also predicted that specialist insect herbivores would perform better on invasive plants, and generalist herbivores would either perform better on the native plants, or there would be no effect of plant origin. To examine whether chemical defenses have been reduced in the introduced range, we measured foliar terpenoid concentrations known to influence both generalist and specialist herbivores (LeQuesne et al., 1986; Cooper-Driver and LeQuesne, 1987). As a by-product of our chemical analysis, we collected data on alkane short-chain hydrocarbons (SCH) concentrations. To test for differences in inducibility of chemical defense between native and invasive *S. gigantea*, we conducted the chemical assays on leaves of both damaged and undamaged plants. Finally, to assess how possible differences in leaf chemistry affected insect herbivores, we performed bioassays with five insect species. We chose a diverse set of insects to test, including both specialist and generalist insects that required four different feeding guilds (leaf chewer, gall-maker, phloem-sap feeder, and xylem-sap feeder).

Methods and Materials

Insect Herbivores Five insects were chosen for study. Two are widespread, generalist insects that frequently become pests in agricultural settings. The meadow spittlebug, *Philaenus spumarius* (Homoptera: Cercopidae), is a xylem-sap feeder that is abundant in both Europe and North America (Weaver and King, 1954). The beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae), is a leaf-chewer that is native to Asia but now occurs worldwide in tropical and temperate areas, with the exception of South America. Both insects are highly polyphagous: *P. spumarius* has been recorded from over 500 host plant species (Weaver and King, 1954) and *S. exigua* uses hosts drawn from over 20 plant families (Greenberg et al., 2001). Whereas *P. spumarius* is frequently found on *S. gigantea* in the field, at least in the US, *S. exigua* is not typically found on *Solidago*. The three other insects studied are all specialists, with each representing a different feeding guild. *Epiblema scudderiana* (Lepidoptera: Tortricidae) is a stem-galler, *Trirhabda virgata* (Coleoptera: Chrysomelidae) is a leaf-chewer, and *Uroleucon nigrotuberculatus* (Homoptera: Aphididae) is a phloem-feeder. All three species feed only on closely related species within the genus *Solidago* (Root and Cappuccino, 1992).

Plant Material S. gigantea is a clonal perennial that is easily propagated from rhizome cuttings. All plants used were grown from rhizomes that were derived from seed collections made in autumn of 1999 from 10 populations in the US and 20 populations in Europe (EU). Seeds were germinated in spring 2000 and transplanted to a field plot at the University of Wisconsin Milwaukee Field Station in Saukville, WI, where they were used for an experiment reported in Meyer et al. (2005). At the conclusion of this experiment in October 2001, rhizomes of all plants were harvested from the field and over-wintered in a refrigerator in closed plastic bags. Thereafter, rhizomes were planted in pots each spring and grown until senescence in the autumn, at which time, the rhizomes were harvested and refrigerated in closed plastic bags over the winter. These methods allowed the same genotypes to be propagated over time. Plants were grown in pots in a 12×12 m screenhouse constructed from aluminum window screen to protect the plants from extraneous insect herbivory. Light levels in the screenhouse were ~70% of those outside. We used a commercial potting mix (Farfard Grow Mix #2, Conrad Farfard, Inc., Agawam, MA, USA), and plants were watered as needed. The only exception was the bioassay with the stem-galler *Epiblema*, where field plots were utilized.

Leaf Secondary Chemistry Plants were grown undamaged in the screenhouse over the 2003 season to minimize any effects of previous treatments. At the end of the 2003 growing season, carry-over effects were assumed to be minimal because European and US plants did not exhibit significant differences in plant height, infructescence mass, or stem diameter (Meyer et al., 2005). On May 4 and 5, 2004, two rhizomes from each of 80 US and 80 EU genotypes were cut to 7–15 cm in length, weighed, and individually potted in 6.6 l pots, for a total of 320 pots. Thinner rhizomes were cut longer in an effort to achieve approximately equal weights. Rhizomes were removed from the refrigerator just before planting and were dormant when planted. Shoots generally emerged in about 7–10 d. Pots were randomly arranged within the screenhouse, with the exception that paired genotypes were kept adjacent to each other. One member of each paired genotype was randomly assigned to receive insect damage, and the other was left undamaged. On May 24, 10–14 larvae of *T. virgata* collected from a local outbreak site were placed on each plant assigned to the damage treatment. Larger plants received more larvae in an effort to achieve approximately equal levels of defoliation. *Trirhabda* larvae are sluggish and do not readily move from plant to plant as long as the pots are not touching. Control (undamaged) plants were examined frequently, and any larvae that had managed to colonize them were removed. Larvae were removed after 18 d of feeding, when defoliation on plants assigned to the damage treatment ranged from 40–100% (mean 77%). Control plants were essentially undamaged (range 0–8%, mean 1% leaf area loss).

Because many of the plants had experienced severe defoliation, and we were concerned about survival, they were given an extended period of recovery before leaves were harvested for chemical analysis. Beginning June 15, plants were sprayed every 7–10 d with the broad spectrum fungicide, Mancozeb, and insecticide, Carbaryl, to control fungal disease and to reduce extraneous herbivory. On July 22 and 27, we switched to the pesticide Camelot (Whitmire Micro-Gen, St. Louis, MO, USA) to control both fungal and bacterial disease. All three pesticides are nonsystemic, and they were effective in keeping extraneous damage to low levels. Spray treatments were discontinued after July 27. On August 16–17, after plants had produced numerous undamaged leaves, five fully expanded leaves from the upper third of the plant stem were harvested from each plant. Leaves were dried at 60°C for 72 hr. Dried leaves were express shipped to Buffalo, NY; upon arrival, leaves were maintained in sealed silica-gel desiccators until processed. Dry leaves were weighed just before chemical analysis. Final sample size was 319 plants because one plant experienced extraneous damage over the growing season.

For chemical analysis, dried leaves from each plant were placed into 15 ml of 70% hexane–30% ethyl acetate solution to which 0.26 mg of nonadecane (internal standard) was added. Leaves were disrupted by using a Polytron tissue homogenizer, centrifuged for 10 min at 2,500 rpm and decanted into 20-ml glass scintillation vials. Vial contents were concentrated under a fume hood to a final volume of ~1.5 ml and analyzed by using a Varian 3900 gas chromatograph (GC) with a flame ionization detector (FID). Compounds were separated on an RTx-5ms column (30 m×0.25 mm id), at an initial temperature of 80°C (3 min) increasing to 280°C at 5°/min. Injector temperature was maintained at 250°C, and the FID kept at 300°C. Sesquiterpene, diterpene, and short chain hydrocarbon FID peaks were grouped by retention times and integrated separately to differentiate concentrations. Identities of the sample peaks were confirmed with an Agilent 6850 GC-5973 MSD by using a similar RTx-5-ms column and the same thermal elution program used for the quantitative runs. The resulting mass spectral ion patterns for each peak were compared to the Agilent NIST98 software database and literature spectra (Anthonsen et al., 1973; Henderson et al., 1973) for compound confirmations.

Insect Bioassays The 2001 field garden was used for the *E. scudderiana* bioassay. This garden was designed to measure colonization of EU and US goldenrods by native insect fauna and consisted of 12 1 m×5 m plots, with half of the plots sprayed to exclude insect herbivores and the other half left unsprayed to measure insect abundances. Each plot had 12 European and 12 US plants drawn from the same 10 US and 20 European populations that were used in the other experiments reported here. Populations were randomly distributed among the plots, and within each plot, European and US plants were alternated. Further details on the design of this garden can be found in Meyer et al. (2005).

Sampling for *E. scudderiana* was restricted to the unsprayed plots of this garden because the spray treatment could affect larval development. There were 135 plants available for colonization by gallmoths (69 EU and 66 US, 6 plots×24 plants/plot minus 9 plants that died). Of these, 51 developed *E. scudderiana* stem galls in 2001, including five plants with double galls. Plants with galls were spread throughout the garden and were not confined to a single location. Taller plants at the time of insect oviposition were more likely to develop galls (Meyer, unpublished data), but there were no significant differences in height between European and US plants (Meyer et al., 2005). *E. scudderiana* showed no preference for European or US plants: 24 US plants and 27 EU plants developed galls, drawn from all 10 US populations and 12 of 20 EU populations. Galls on three plants were empty (two EU and one US), so the final sample size was 23 US plants and 25 EU plants.

All visible galls were collected from the garden on Sept. 27, 2001, and caterpillars were extracted from them. Galls collected in fall generally contained a single caterpillar that hatched in spring and spent the summer feeding within the gall. The caterpillar overwinters in the gall and pupates the following spring. Thus, galls collected in fall contain fully grown larvae. Caterpillars were frozen, dried at 70°C for 48 hr, and then weighed to the nearest 0.01 mg.

The *P. spumarius* bioassay was conducted in 2002 by using rhizomes derived from the 2001 field garden (described in Meyer et al., 2005). This garden consisted of paired plots that were either sprayed with insecticide or left unsprayed in 2000 and 2001. On April 17, 2002, rhizomes from eight US and eight EU populations were removed from the refrigerator, cut to 10 cm in length, and planted in 7.6-l pots. The original 10 US and 20 EU populations came from 6 EU countries and 10 US states; populations included in the *Philaenus* trial were selected so that all countries and states were represented. Four rhizomes were planted from each population: two each from previously sprayed and previously unsprayed plots. Two spittlebug nymphs collected from a local population were placed on each experimental plant with a fine-hair paintbrush on May 15. Plants were monitored daily until the nymphs were nearing their final molt. All insects were collected from the experimental plants on June 17, after a single adult spittlebug was seen in the screenhouse. Insects were frozen overnight, dried at 65°C for 48 hr, and then, weighed to the nearest 0.1 mg. Final sample size was 58 plants (two rhizomes failed to emerge and four plants, 2 US and 2 EU, lacked spittlebugs at harvest).

Bioassays for *T. virgata*, *S. exigua*, and *U. nigrotuberculatus* were conducted in 2005 with rhizomes harvested from the plants used in 2004 to measure leaf secondary chemistry. The insect bioassays included plants that had been damaged or undamaged in the previous year, to look for carry-over effects of damage on insect performance. Three rhizomes from each of the 320 plants used in 2004 were removed from the refrigerator, cut to ~1 g (range 0.8±1.2 g) and individually potted in 4.16-l pots. The pots were arranged in three sets within the screenhouse. Plants were randomly arranged within each set, except that the paired previously damaged and previously undamaged members of each genotype were kept adjacent to each other. One set of plants was used for each of the 3 insects.

Plants for the *T. virgata* bioassay were planted on May 4-5, and plants for the *S. exigua* and *U. nigrotuberculatus* bioassays were planted on May 17-19. Final sample sizes for plants were less than the 320 originally planted because a few rhizomes failed to emerge in each set of plants.

For the *T. virgata* bioassay, larvae were collected from a local outbreak site on June 6. Two first-instars were placed on each plant in the first set. The insects fed for 14 d, until just before. Larvae were then transferred to individual petri dishes with moistened filter paper and monitored until pupation. Fresh excised leaves from the corresponding host plant were added daily until larvae discontinued feeding. Fresh pupal mass was measured to the nearest 0.1 mg. At least one insect survived to pupation on 137 out of 153 European plants, and 142 out of 155 US plants, for a final sample size of 279.

The *S. exigua* assay was conducted with eggs obtained from the United States Department of Agriculture (USDA) insect-rearing facility in Stoneville, MS, USA. Five to 10 first instars were collected by using a fine-hair paintbrush and transferred to 237-ml plastic cups with lids. We started with multiple caterpillars because we expected some mortality and wanted to ensure that each plant had at least one surviving caterpillar. Caterpillars were fed on excised leaves from the second set of plants, with fresh leaves provided at least every third day. After 7 d, cups were reduced to five randomly chosen caterpillars to reduce the demand for leaf material to feed the growing caterpillars, and after 14 d, survivors were separated into individual cups. The feeding trial was discontinued when the first pupa appeared on day 21. Fresh mass of all surviving insects was measured to the nearest 0.1 mg. The majority of insects were still caterpillars at the time they were weighed, as only two pupae were found. Over the 3-wk bioassay, a total of 85 plants lost all caterpillars to mortality. US plants seemed somewhat more likely to lose all of their caterpillars than EU plants (49/156 US plants had mortality of all caterpillars, compared to 36/152 European plants), but this trend was not significant (χ^2=2.3, *df*=1, *P*=0.13).

On July 29, 2005, we observed that the third set of plants in the screenhouse had been voluntarily colonized by *U. nigrotuberculatus* aphids. We used this as a natural experiment and monitored the population growth of aphids on the experimental plants. The number of aphids on each of 303 plants was counted on August 29, after approximately 1 mo of population growth. European and US plants were equally likely to support aphids (58/150 European plants and 63/153 US plants had aphid colonies).

Data Analysis All data were tested for normality and homogeneity of variance before analysis; chemical yields, *Spodoptera* caterpillar mass, and aphid counts were log transformed to meet test assumptions. Analyses were performed by using SAS (version 8.2; SAS Institute, 2001).

The effects of continent of origin and damage on sesquiterpene, diterpene, and short chain hydrocarbon chemical yields were analyzed by using nested analysis of variance. Continental origin and damage were treated as fixed effects, and population of origin was included in the model as a nested, random effect. *F*-statistics were constructed by following methods detailed by Neter et al. (1996). Insect performance data for *Trirhabda* beetles, *Spodoptera* beet armyworms, *Uroleucon* aphids, and *Philaenus* spittlebugs were initially analyzed with a model that included continent of origin and previous damage to the host plant as fixed terms, and population of origin as a nested random term. These analyses showed that there were no significant effects ($P>0.10$) of previous damage on insect performance for any of the four insects, so previous damage was dropped from the model and pooled into the error term. For *Epiblema*, insect performance data were analyzed by using a one-way ANOVA that included only continent of origin. This insect bioassay did

not incorporate the effects of previous damage, and it lacked replication within some populations, so the nested ANOVA could not be used.

Insect mass per plant was used to measure performance for *Trirhabda*, *Spodoptera*, *Philaenus*, and *Epiblema*. For cases where there was more than one surviving insect per plant, the mean mass of the surviving insects was used for *Trirhabda*, *Philaenus*, and *Epiblema*, and the mass of the largest surviving individual was used for *Spodoptera*. The mass of the largest surviving individual was used for *Spodoptera* because these individuals were assumed to be closest to pupation, and thus, near a similar stage of development to *Trirhabda* and *Epiblema*. Insect number per plant was used for *Uroleucon* aphids. Female *Trirhabda* pupae were significantly larger than male pupae, but inclusion of beetle sex in the model did not alter conclusions, and these analyses are not shown.

The effects of sesquiterpene, diterpene, and short-chain hydrocarbon foliar concentrations on performance of *Trirhabda* and *Spodoptera* were analyzed by using analysis of covariance and regression analyses. We chose to focus on the two leaf-feeding insects for these analyses because it is not clear to what extent chemicals measured in the leaves would affect the performance of the aphid or spittlebug, which feed on plant saps. The chemical yield measured for each plant in 2004 was matched with the performance of insects grown on plants derived from the rhizomes of that plant in 2005. Thus, chemistry was not measured on the same leaves on which the insects fed, but these analyses can reveal relationships between insect growth and plant chemistry, if there is a strong genetic component to leaf secondary chemistry.

The analysis of covariance (ANCOVA) model included chemical yield as a continuous variable, continental origin as a fixed effect, population nested within continental origin as a random effect, and all interactions. A significant effect of chemical yield indicates that insect performance is affected by varying concentrations of that chemical. We predicted that leaf-chewing insect performance would be negatively affected by increasing concentrations of sesquiterpenes, diterpenes, or SCH. Continental origin was included in the model to detect whether or not insect responses to plant chemistry depended on the origin of the plant. This might occur if the composition of each class of chemicals varied between ranges and would be shown by a significant interaction between continent and chemical yield. The population term was included in the model to account for variation within the range on each continent. For cases where the population term or interactions with population were not significant ($P>0.10$), the population term was removed from the model and pooled into the error term. Regressions that related the levels of secondary chemicals to insect performance were run when the ANCOVA indicated a significant effect of chemical yield. If the ANCOVA revealed a significant or marginally significant ($P<0.10$) interaction between chemical yield and plant origin, regressions between insect performance and chemical yield were run separately for each continental source.

Results

The continental origin of the plants did not significantly affect sesquiterpene, diterpene, or SCH (Table 1, Fig. 1), but there was highly significant variation across populations for all three chemicals (Table 1). Whereas mean diterpene concentrations were 11% higher in US compared to European plants (pooled across damaged and undamaged plants), this difference was not significant because of high levels of variation among populations within each continent. Damage did not affect sesquiterpene or diterpene yield, but did significantly reduce SCH concentrations (Table 1, Fig. 1). Short chain hydrocarbon concentrations were

Table 1 Effect of insect damage, continental source and population on chemical concentration (LN transformed), tested by GLM

Source of Variation	C15			C20		SCH	
	Df	MS	*F*	MS	*F*	MS	*F*
Damage Treatment	1	0.1799	1.29	0.1465	2.95*	9.7681	**26.92****
Seed source	1	0.3860	0.40	0.9962	1.39	8.9247	3.30*
Population(source)	28	0.9631	**2.83****	0.7162	**3.16****	2.7035	**7.45****
Damage**Source	1	0.2220	1.59	0.0191	0.39	2.8401	0.01
Damage**Population(source)	28	0.1396	0.41	0.0496	0.22	0.1346	0.37
Error	259	0.3406		0.2269		0.3628	

Bold fonts indicate a significant difference

C15 Sesquiterpenes, *C20* diterpenes, and *SCH* straight chain hydrocarbons

*$P \leq 0.1$,

****P*≤0.05**

*****P*≤0.01**

******P*≤0.001**

28% lower in damaged plants compared to undamaged plants (pooled across European and US plants) (Fig. 1).

Of the five insects tested, only the generalist leaf-chewer *Spodoptera* performed significantly better on plants from the invasive range (Table 2): mean insect mass was 37% lower on US compared to European plants (Table 3). In addition, *Spodoptera* growth response to European host plants exhibited greater variability than to US host plants as evidenced by the greater standard deviation within the EU plants (Levene's test: s^2 ratio= 15.607; $P<0.001$). Coefficients of variation for *Spodoptera* growth ranged from 17.06 to 75.90% among European populations and from 18.74 to 61.97% among US populations. The other four insects performed equally well on plants from the invasive range and the native range (Tables 2 and 3). Population effects were significant for the generalist leaf-chewer *Spodoptera*, the specialist leaf-chewer *Trirhabda*, and the specialist aphid *Uroleucon*, whereas growth of the generalist xylem-sap feeder *Philaenus* was not influenced by the host plant's population of origin. Population effects could not be tested for the stem-gall maker, *Epiblema*, because of small sample size and lack of replication within some populations.

The effect of increasing concentrations of foliar chemicals on performance of the two leaf-chewing insects differed for the generalist *Spodoptera* and the specialist *Trirhabda*. Increasing concentrations of diterpenes decreased the growth of *Spodoptera* caterpillars, and there was no interaction with continental origin (Table 4). Regression analysis revealed a significant negative relationship between diterpene concentration and *Spodoptera* mass (Fig. 2, slope=−0.007, $P=0.002$, $R^2=0.04$). In contrast, *Trirhabda* growth was not affected by foliar diterpene concentration (Table 4, Fig. 2). While the interaction between diterpene yield and continental origin was marginally significant for *Trirhabda* ($P=0.08$), an examination of the relationship between diterpene yield and beetle mass for European and US plants separately did not reveal any significant effects (EU: $P=0.27$, US: $P=0.17$, *P* value of slope in simple regression). However, *Trirhabda* growth was affected by foliar short chain hydrocarbon concentrations (Table 4). The effect of short chain hydrocarbon yield on *Trirhabda* growth seemed to depend on the continental origin of the plants ($P=0.059$), so simple regressions were run for EU and US plants separately (Fig. 3). *Trirhabda* growth

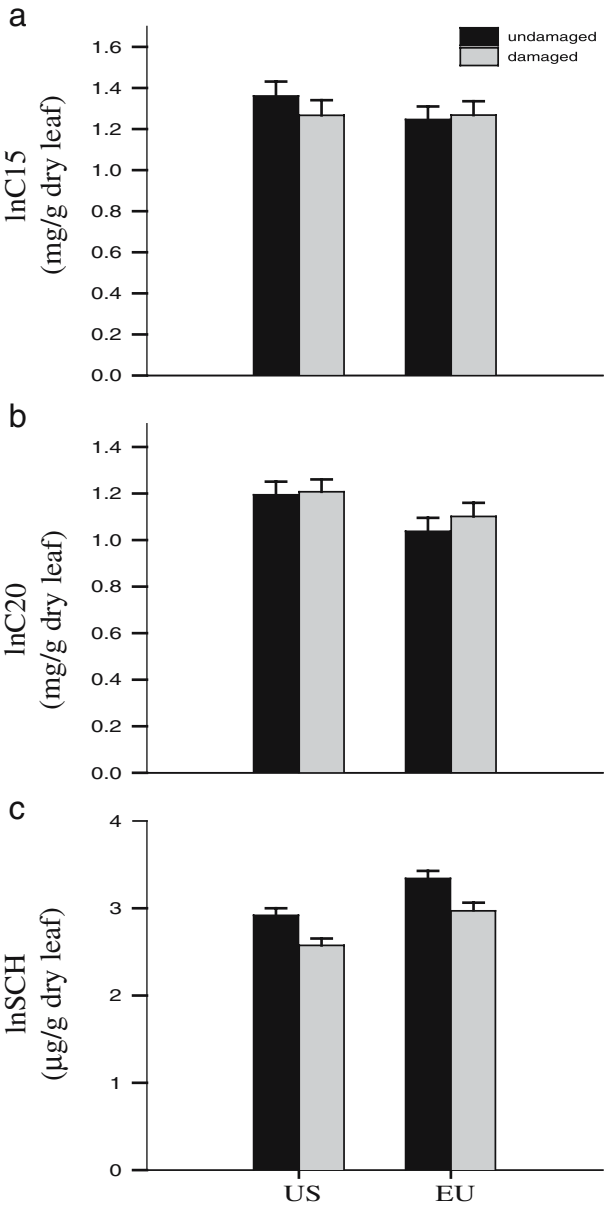


Fig. 1 Chemical yields of **a** Sesquiterpene (C15) yields, **b** diterpene (C20) yields, and **c** straight chain hydrocarbons (SCH) in damaged and undamaged (mean± SE) leaves of end of season plants of US and European (EU) populations of *Solidago gigantea*, grown under common screenhouse conditions at the University of Wisconsin—Milwaukee Field Station, Saukville, WI. *Ln* transformed values are shown

declined with increasing short chain hydrocarbon concentrations, but only for US host plants (EU: slope=0.002, P=0.09, R^2=0.02, US: slope=−0.004, P=0.002, R^2=0.07, simple regressions). *Spodoptera* growth was not significantly affected by short chain hydrocarbon yield, continent of origin, or their interaction (Table 4); however, at concentrations of >0.75 mg/g dry leaf, only one out of eight US plants supported any surviving caterpillars, whereas 13 out of 15 EU plants had at least one surviving caterpillar. Increased concentrations of sesquiterpenes did not affect the performance of either *Trirhabda* or *Spodoptera*, and there were no interactions (Table 4).

Table 2 Effect of plant continental source and population on insect growth (*Philaenus spumarius* spittle bugs, *Spodoptera exigua* armyworms, *Uroleucon nigrotuberculatus* aphids, *Trirhabda virgata* beetles, and *Epiblema scudderiana* gall makers), tested by GLM

Source of variation	Generalist Herbivores						Specialist Herbivores								
	Philaenus			Ln (*Spodoptera*)			Ln (*Uroleucon*)			*Trirhabda*			*Epiblema*		
	df	MS	*F*	*df*	MS	*F*	*df*	MS	*F*	*df*	MS	*F*	*df*	MS	*F*
Seed source	1	6.05×10^{-7}	2.25	1	12.35	**5.73****	1	0.52	0.05	1	1.32×10^{-6}	0.0808	1	79.91	0.63
Population (source)	14	2.74×10^{-7}	0.66	28	2.64	**2.65****	28	11.16	**2.60****	28	33.63×10^{-6}	**2.13****			
Error	42	1.74×10^{-5}		193	0.99		273	4.29		249	15.81×10^{-6}		46	126.91	

Bold fonts indicate a significant difference.

*$P\leq0.1$

****P*≤0.05**

*****P*≤0.01**

******P*≤0.001**

Table 3 Means±SE mass (mg) *Philaenus spumarius* spittle bug nymphs, *Spodoptera exigua* armyworm caterpillars, *Trirhabda virgata* beetle pupae, and *Epiblema scudderiana* galling caterpillars and mean±SE number of individual *Uroleucon nigrotuberculatus* aphids reared on European invasive and US native populations of *Solidago gigantea*

Seed Source	Generalists				Specialists					
	N	*Philaenus*	*N*	*Spodoptera*	*N*	*Uroleucon*	*N*	*Trirhabda*	*N*	*Epiblema*
EU	29	4.20±0.11	116	**62.02±6.02**	150	66.35±9.07	137	34.67±0.37	25	16.89±2.25
US	29	4.04±0.12	107	**39.07±4.10**	153	63.77±9.05	142	34.75±0.34	23	19.48±2.35

Bold fonts indicate a difference (p=0.02) in insect response between invasive and native food sources.

Discussion

The results of our study do not provide strong support for the EICA hypothesis. There was no evidence that concentrations of sesquiterpenes or diterpenes, chemicals in mature plants associated with defense, were lower in invasive plants from Europe, whether plants were

Table 4 Effect of seed source and population on insect growth (*Spodoptera exigua* armyworm caterpillars and *Trirhabda virgata* beetle pupae) covaried by chemical yield (*C15* Sesquiterpenes, *C20diterpenes*, and *SCH* straight chain hydrocarbons), tested by GLM

Source of Variation	Generalist			Specialist		
	Spodoptera			Trirhabda		
	df	MS	*F*	*df*	MS	*F*
C15 yield	1	5.58×10^{-5}	0.02	1	6.34×10^{-5}	3.62*
Seed source	1	0.003	0.73	1	1.67×10^{-6}	0.1
Population (source)	27	0.004	**1.66****			
C15**source	1	2.64×10^{-4}	0.1	1	3.26×10^{-6}	0.19
C15**Population (source)	27	0.003	1.24			
Error	164	0.003		275	1.75×10^{-5}	
C20 yield	1	0.0249	**8.50*****	1	8.000×10^{-8}	0
Seed source	1	0.0189	**6.45****	1	4.121×10^{-5}	2.35
Population (source)						
C20**source	1	0.0068	2.3	1	5.384×10^{-5}	3.07*
C20**Population (source)						
Error	219	0.0029		275	1.75×10^{-5}	
SCH yield	1	0.0008	0.34	1	3.992×10^{-6}	0.28
Seed source	1	0.0009	0.17	1	77.768×10^{-6}	3.04*
Population (source)	27	0.0055	**2.26******	28	25.552×10^{-6}	**1.81*****
SCH**source	1	0.0018	0.74	1	50.597×10^{-6}	3.59*
SCH**Population (source)	27	0.0037	1.51*	28	24.970×10^{-6}	**1.77****
Error	164	0.0024		219	14.085×10^{-6}	

Nested population terms pooled into error term if model indicated ns at p>0.10.

Bold fonts indicate a significant difference.

*$P\leq0.1$

****P*≤0.05**

*****P*≤0.01**

******P*≤0.001**

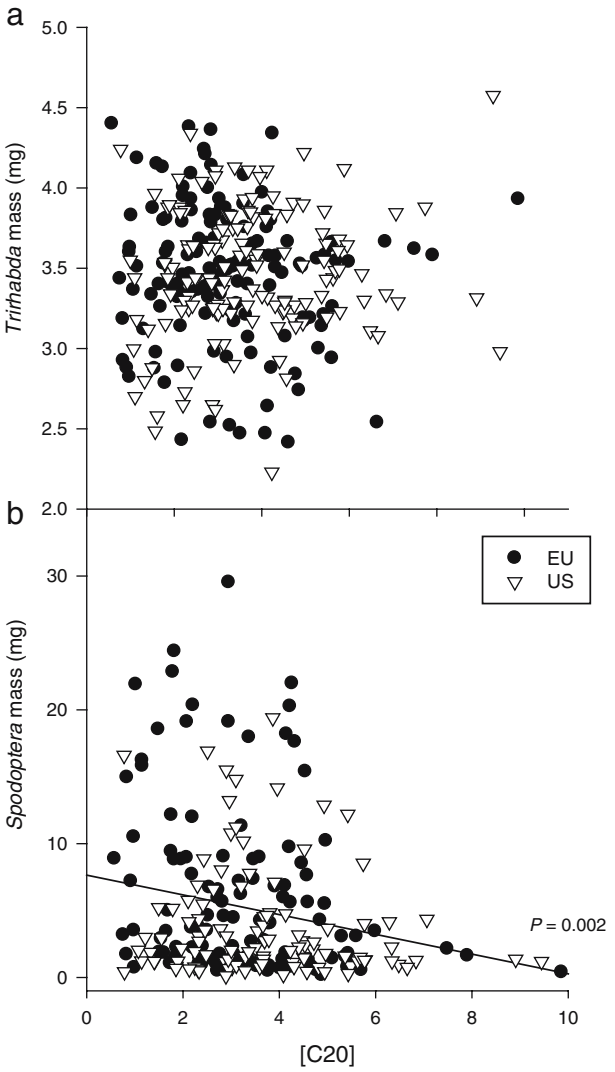


Fig. 2 Effects of diterpene (C20) yield (mg/g dry leaf) of US native and European (EU) invasive *Solidago gigantea* plants on **a** *Trirhabda virgata* pupal mass (mg) and **b** *Spodoptera exigua* larval mass at 21 d (mg). The *line* shown in **b** is from a simple regression relating *S. exigua* mass to C20 concentration, and the *P*-value is for the slope of the line

damaged or undamaged. Similarly, all three of the specialist insects that we tested performed equally well on plants from the invasive and the native range. Under the predictions of the EICA hypothesis, reduced host plant resistance to all three insects should have been seen in European plants, as none of these insects are found in Europe. In particular, we expected to see reduced defenses against *Trirhabda* beetles because this insect is among the most abundant herbivores on *Solidago* in North America and exerts a strong selective force on its host plant (Root and Cappuccino, 1992). *Trirhabda* beetles can reach outbreak densities where entire fields of goldenrod are completely defoliated, and plant mortality after such outbreaks has been documented (McBrien et al., 1983). We also expected to see altered performance of the *Epiblema* stem-galler on European plants because of the intimate relationship between this internally feeding insect and its host plant. Reduced defenses against the specialist aphid *Uroleucon* were perhaps less likely because

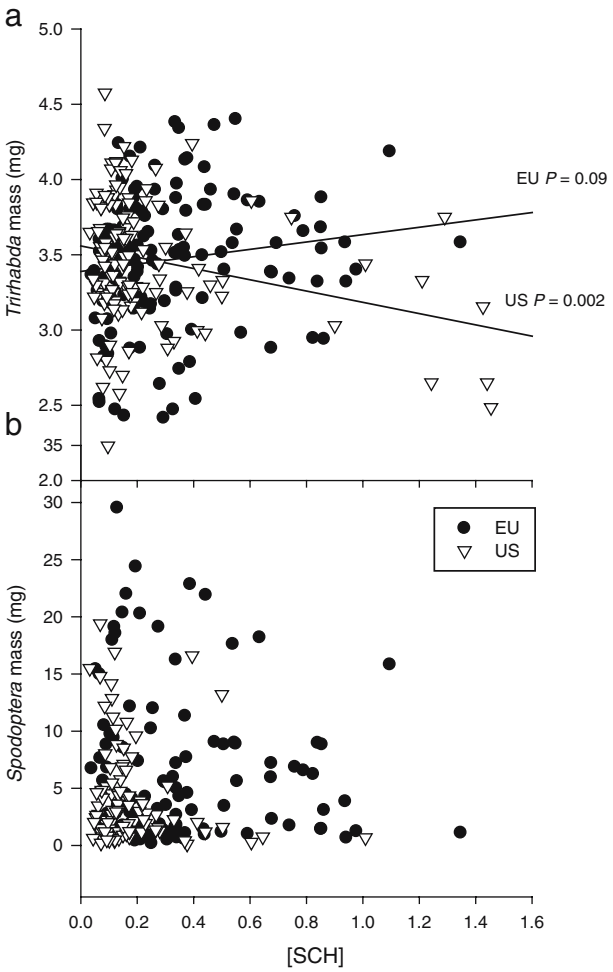


Fig. 3 Effects of straight chain hydrocarbon (SCH) yield (mg/g dry leaf) of US native and European (EU) invasive *Solidago gigantea* plants on **a** *Trirhabda virgata* pupal mass (mg) and **b** *Spodoptera exigua* larval mass at 21 d (mg). The lines shown in **a** are from simple regressions relating *T. virgata* mass to SCH concentration and the *P*-values are for the slopes of the lines

aphid feeding is less damaging to the host plant than other types of herbivore feeding (Meyer, 1993; Meyer and Root, 1993). However, aphids may respond to plant characteristics that have been selected for by other herbivores (Pilson, 1992). We expected that European plants would retain defenses against the generalist spittlebug, *Philaenus*, because this insect would be encountered on both continents. Native to Europe, *Philaenus* is also abundant in North America, where it has been introduced and has become an invasive crop pest (Weaver and King, 1954).

Nevertheless, our study did reveal changed defenses in invasive plants, as the generalist leaf-chewer *Spodoptera* performed significantly better on European plants. This result fits the prediction of the EICA hypothesis that defenses should be lower in the introduced range, but is counter to the corollary that invasive plants should maintain sufficient defenses to deter generalist herbivores (Maron and Vilà, 2001; Joshi and Vrieling, 2005). While the relationships between generalist insects, specialist insects, and invasive plants are just beginning to be explored, studies to date have shown that invasive plants tend to retain defenses against generalists (Bossdorf et al., 2004; Joshi and Vrieling, 2005; Leger and Forister, 2005; Hansen et al., 2006). Our results, revealing retention of defenses against

specialist insects and loss of defenses against a generalist insect, are difficult to interpret. It is possible that the defenses that *Spodoptera* were responding to are also effective against some other natural enemy that may have been absent in the introduced range. *Spodoptera* larval mass declined with increasing foliar diterpene concentration, suggesting that these caterpillars were responding to host plant secondary chemistry. While these diterpenes were not effective against the specialist *Trirhabda* beetles (i.e., no loss in larval mass with increasing foliar concentration), they could be acting against some other natural enemy, such as foliar pathogens. Previous research with *S. gigantea* has shown that European plants are more susceptible than US plants to two foliar pathogens (Meyer et al., 2005), a rust fungus and a bacterial leaf blight, that are not known to occur widely on plants in Europe. Many secondary plant metabolites have traditionally been regarded as compounds that are specifically active against either herbivores *or* pathogens (Harborne, 1993); however, several classes can affect the performance of both herbivores and pathogens (Barbosa, 1991; Krischik, 1991; Karban and Baldwin, 1997) and are, therefore, considered to function as "generalized" defense compounds (Krischik et al., 1991). Biere et al. (2004) found that the terpene derivatives, such as iridoid glycosides, deterred feeding by generalist herbivores and reduced spore production by fungal pathogens.

There was no evidence for enhanced levels of secondary chemicals after herbivore damage. This result should be interpreted with caution, as several features of our experimental design may have weakened our ability to detect induced defenses. First, leaves for chemical analysis were harvested approximately 9 weeks after herbivore damage. We expected that defenses directed against larval *Trirhabda* might still be present later in the season because larval and adult *T. virgata* feed on the same host plant. In the field, defoliation by larvae is usually followed by adult damage that can last through the summer. While we found no evidence that feeding by larvae changed host quality later in the season, the possibility remains that chemical changes may have occurred over shorter time scales than utilized in our experiment. Second, the high levels of defoliation experienced by plants in our experiment may have limited their ability to boost their level of defense. The plants were left with little remaining leaf area after the insects were removed, and investing in growth may have had priority over enhancing defenses as the plants recovered. Third, the plants might have responded differently if we had used a generalist insect rather than a specialist. Finally, we looked at broad chemical classes and did not examine individual compounds. Previous work with *S. gigantea* has shown that the occurrence of major sesquiterpene and diterpene peaks is similar for European and US plants, at least when plants are undamaged (Johnson and Meyer, unpublished data).

Our experiment did reveal effects of damage on SCH, but in this case, concentrations were reduced by herbivory, with European and US plants responding similarly. The role of SCH in plant defense is not clear, but they are known to function as volatile signals that can attract insects to plants. For example, SCH extracted from insects (Lewis et al., 1975; Padmavathi and Paul, 1998) or leaves and flowers of plants (Tandon and Bakthavatsalam, 2005) are a component of odor volatiles that increase the recruitment of *Trichogramma* sp. parasitoids; SCH also attract pseudo-copulating bees to pollinate orchids (Paxton and Tengo, 2001). Our results suggest a more direct role for SCH in plant defense, as *Trirhabda* beetle growth declined, and *Spodoptera* mortality may be greater with increasing SCH concentration, but only for US plants. It is also possible that SCH were acting as a volatile signal of some kind in our experiment because the decreased concentrations in damaged plants suggests that, in the presence of insect herbivores, these compounds were released or depleted in some way. Our experiment was not designed to detect any effects of volatile signals on herbivore natural enemies, so this possibility remains unexplored. One intriguing

potential explanation for our failure to detect induced chemical defenses in this experiment is the possibility that volatile signals from damaged plants may have induced neighboring undamaged plants to increase their chemical defenses. While we are unaware of any work documenting volatile signals in *Solidago*, it is now well-accepted that volatile organic compounds can induce defenses in neighboring, undamaged plants (see Baldwin et al., 2006 for review). This might have been possible in our experiment as damaged and undamaged plants were grown intermixed in close proximity, and defoliation levels were high.

Previous work on the secondary chemistry of *S. gigantea* has shown significantly greater levels of diterpenes in native plants compared to invasive plants, as the EICA hypothesis predicts. In an experiment that used undamaged plants grown from seed, Johnson and Meyer found that diterpene concentrations of US plants were 44.5% greater than those of European plants (unpublished data). While the trend was the same in the experiment reported here, differences between EU and US plants were much smaller (11%) and not significant. Diterpene concentrations measured in the present experiment were also more than twice as high as those in the other study (mean calculated across all plants=3.5 mg/g dry leaf this study, vs. 1.5 mg/g dry leaf, Johnson and Meyer, unpublished data). Diterpene concentrations measured in seedling plants unexposed to herbivores, as in the other study, represent constitutive levels of defenses. The increased amounts seen in the current experiment are consistent with the idea that all plants were induced to increase their chemical defenses, either directly via herbivore feeding or indirectly via a volatile signal. While we cannot rule out other explanations, such as different energy reserves available to seedlings and rhizome-derived plants, it is possible that we did not detect differences in chemistry between European and US plants or effects on specialist herbivores because all plants were induced. Recently, Cipollini et al. (2005) reported that invasive populations of *Alliaria petiolata* tended to have lower constitutive levels but greater inducibility of glucosinolates. The possibility that other introduced plants retain their ability to induce defenses while maintaining lower constitutive levels of defense deserves further exploration.

Our study represents one of the most complete tests of the defense predictions of the EICA hypothesis to date, as it incorporates direct measures of secondary chemistry along with bioassays of both generalist and specialist insects across a greater spectrum of feeding guilds than have been tested previously. *S. gigantea* has been present in Europe for over 250 years (Weber, 1998) and experiences little herbivory there (Jakobs et al., 2004), so the prerequisites that might lead to an evolutionary loss of defenses seem to be in place. We found little support for two major predictions of the EICA hypothesis: that invasive plants should have lower levels of chemical defenses and support better growth of specialist insects. However, our results do reveal genetically based changes in the defenses of European plants, as we found increased growth of *Spodoptera* on invasive plants, and previous work has revealed lower levels of diterpenes and increased susceptibility to pathogens in invasive plants (Johnson and Meyer, unpublished data; Meyer et al., 2005). These results suggest that changes in plant defenses in the introduced range of *S. gigantea* are more complex than would be expected under the current predictions of the EICA hypothesis, even including extensions incorporating the difference between specialist and generalist insects (Müller-Schärer et al., 2004; Joshi and Vrieling, 2005; Leger and Forister, 2005; Hansen et al., 2006). Our study also demonstrated strong population effects for all three chemical classes that we measured and for three of the four insects where we had sufficient replication to test for differences among populations. Our results suggest that future studies testing the defense predictions of the EICA hypothesis should incorporate a broad spatial scale and include a diversity of herbivores. In addition, the possibility that invasive plants retain the ability to induce defenses against herbivores should be further explored.

Acknowledgements We thank Terry Bott and Lou Nelson for help in the garden. Special thanks to Jane Clare and Stephen Sanders. Two anonymous reviewers provided comments that improved the manuscript. Financial support was provided by NSF grant DEB-0315430 to G. Meyer and DEB-035127 to R. Johnson.

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J Chem Ecol (2007) 33:801–815
DOI 10.1007/s10886-007-9270-9

Methyl 2,4,6-decatrienoates Attract Stink Bugs and Tachinid Parasitoids

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Received: 3 October 2006 / Accepted: 21 February 2007 /
Published online: 3 March 2007
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Abstract *Halyomorpha halys* (Stål) (Pentatomidae), called the brown marmorated stink bug (BMSB), is a newly invasive species in the eastern USA that is rapidly spreading from the original point of establishment in Allentown, PA. In its native range, the BMSB is reportedly attracted to methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate, the male-produced pheromone of another pentatomid common in eastern Asia, *Plautia stali* Scott. In North America, *Thyanta* spp. are the only pentatomids known to produce methyl 2,4,6-decatrienoate [the (*E*,*Z*,*Z*)-isomer] as part of their pheromones. Methyl 2,4,6-decatrienoates were field-tested in Maryland to monitor the spread of the BMSB and to explore the possibility that *Thyanta* spp. are an alternate host for parasitic tachinid flies that use stink bug pheromones as host-finding kairomones. Here we report the first captures of adult and nymph BMSBs in traps baited with methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate in central Maryland and present data verifying that the tachinid, *Euclytia flava* (Townsend), exploits methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate as a kairomone. We also report the unexpected finding that various isomers of methyl 2,4,6-decatrienoate attract *Acrosternum hilare* (Say), although this bug apparently does not produce methyl decatrienoates. Other stink bugs and tachinids native to North America were also attracted to methyl 2,4,6-decatrienoates. These data indicate there are Heteroptera in North America in addition to *Thyanta* spp. that probably use methyl 2,4,6-decatrienoates as pheromones. The evidence that some pentatomids exploit the pheromones of other true bugs as kairomones to find food or to congregate as a passive defense against tachinid parasitism is discussed.

Keywords Aggregation pheromone · Kairomone · Selfish herd · Methyl decadienoate · *Acrosternum* · *Thyanta* · *Podisus* · *Halyomorpha* · *Banasa* · *Euclytia* · *Gymnosoma* · Hemiptera · Heteroptera · Pentatomidae

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Introduction

Much has been learned about stink bug communication, both chemical and acoustic, since *Thyanta custator* (F.) (Pentatomidae) rained from the sky in a line across Kansas on October 14, 1939, requiring truck-loads of bugs to be hauled off in the subsequent cleanup (Wilbur, 1939). It is now known that pentatomid males produce pheromones attractive to conspecific females, males, and nymphs, as well as tachinid flies and other parasitoids (Aldrich, 1988b, 1995; McBrien and Millar, 1999). Ultimately, adult stink bugs recognize and court each other acoustically (Çokl and Virant-Doberley, 2003). Males of *Thyanta* spp., for example, produce methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate with (Millar, 1997; McBrien et al., 2002b) or without particular sesquiterpenes (Moraes et al., 2005) that attract a mate, while at close range, a dialog of substrate vibrations facilitates species recognition and isolation (McBrien et al., 2002a). The methyl 2,4,6-decatrienoate pheromone theme has also been reported for one other pentatomid bug native to eastern Asia, *Plautia stali* Scott, whose males produce methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate (Sugie et al., 1996).

Halyomorpha halys (Stål) (Pentatomidae), called the brown marmorated stink bug (BMSB), is a newly invasive species in the eastern USA (Hoebeke and Carter, 2003) that is rapidly spreading from its original point of establishment in Allentown, PA. The pheromone for *H. halys* is unknown, but in Japan, this bug is reportedly cross attracted to the pheromone of *P. stali* (Tada et al., 2001a, b).

Perhaps the best known pheromone system among the true bugs (Hemiptera: Heteroptera) is that of the spined soldier bug, *Podisus maculiventris* (Say) (Pentatomidae) (Aldrich et al., 1978, 1984a, b, c; Aldrich, 1985; Sant'Ana et al., 1997). Males of this predacious bug produce an attractant pheromone consisting of two essential components, (*E*)-2-hexenal and α-terpineol. Among various parasitoids that exploit this pheromone as a host-finding kairomone, the most commonly encountered species is the tachinid fly, *Euclytia flava* (Townsend). However, *E. flava* is seldom captured in traps baited with synthetic *P. maculiventris* pheromone late in the summer or early fall, and this fly almost never emerges from over wintering spined soldier bugs caught in pheromone-baited traps in the spring. *Euclytia flava* has been reared from *Thyanta* spp. field-collected from late July through September (Eger and Ables, 1981; Jones et al., 1996), suggesting that *Thyanta* bugs might be a late-season alternate host for *E. flava*.

Thus, we became interested in field-testing methyl 2,4,6-decatrienoates for two reasons: (1) in an effort to monitor the spread of the BMSB, *H. halys*, and (2) to explore the possibility that *Thyanta* spp. are an alternate host for the tachinid fly, *E. flava*. Here, we report data pertinent to these two avenues of investigation but also report the unexpected finding that various isomers of methyl 2,4,6-decatrienoate are potent attractants for native stink bugs and their tachinid parasitoids although efforts to isolate these compounds from the bugs themselves have thus far failed.

Methods and Materials

Chemical Standards and Treatments Standards of the following were purchased: (*E*)-2-hexenal, methyl (*E*,*Z*)-2,4-decadienoate (Bedoukian Research, Danbury, CT, USA); α-terpineol (Fisher Scientific, Fair Lawn, NJ, USA); and bisabolene (International Flavors & Fragrances, Union Beach, NJ, USA). Methyl (*E*,*Z*,*Z*)-, (*Z*,*E*,*Z*)-, and (*E*,*E*,*Z*)-2,4,6-decatrienoates were synthesized as previously described (Khrimian, 2005), as were (4*S*)-*cis*- and (4*S*)-*trans*-(*Z*)-α-bisabolene epoxides [(*Z*)-(1*R*,2*S*,4*S*)-4-(1′,5′-dimethyl 1′,4′-hexadienyl)-1,

2-epoxy-1-methylcyclohexane and (*Z*)-(1*S*,2*R*,4*S*)-4-(1′,5′-dimethyl 1′,4′-hexadienyl)-1,2-epoxy-1-methylcyclohexane, respectively] (Chen et al., 2000). Zingiberene [5-(1,5-dimethyl-4-hexenyl)-2-methyl-1,3-cyclohexadiene] was the most convenient sesquiterpene to use for the pheromone because this compound could be isolated from ginger essential oil by using the procedure of Millar (1998). Structures of known pheromone molecules that were tested are shown in Fig. 1.

Lures for the initial experiment (Exp. 1) included treatments mimicking the natural pheromones of *P. maculiventris* (Aldrich et al., 1984a) and *T. custator accerra* McAtee (McBrien et al., 2002b), with five replicates per treatment prepared as follows. (*E*)-2-hexenal and racemic α-terpineol were mixed in a molar ratio of 1:0.843. Batches of 40 ml of pheromone were prepared by combining 17.6 ml hexenal and 21.4 ml of α-terpineol. A 20% (vol/wt) formulation of pheromone in plasticized polyvinyl chloride (PVC) was prepared by mixing 107 g of powdered PVC (Tenneco, Piscataway, NJ, USA) with 53 g of dioctyl phthalate (Aldrich Chemicals, Milwaukee, WI, USA) (Fitzgerald et al., 1973). The

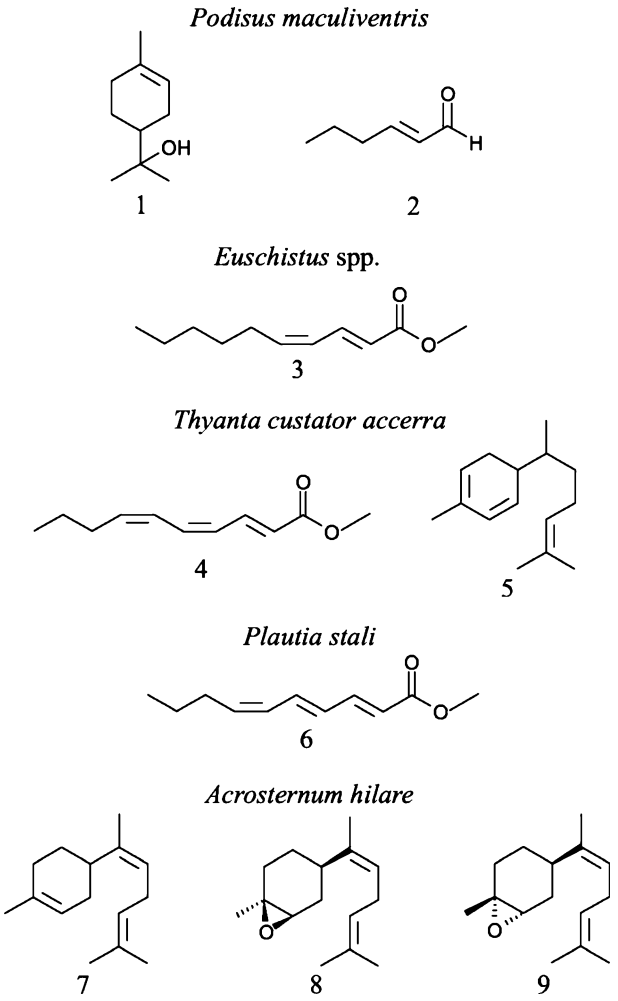


Fig. 1 Chemical compounds tested and the pentatomid species with which the compounds have been associated: *1*, α-terpineol; *2*, (*E*)-2-hexenal; *3*, methyl (*E*,*Z*)-2,4-decadienoate; *4*, methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate; *5*, zingiberene; *6*, methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate; *7*, (*Z*)-α-bisabolene; *8*, *cis*-(*Z*)-α-bisabolene epoxide; and *9*, *trans*-(*Z*)-α-bisabolene epoxide

pheromone–PVC mixture was poured into test tubes (to the top; 16×100 mm, Kimble, Elmira, NY, USA), heated in an oven at 110°C for 30 min, removed from the tubes, and stored in sealed vials in a freezer until use (treatment = "H+α-T"). Approximately 1-g slices of the H+α-T treatment in PVC were used to bait traps.

In Exp. 1, lures for treatments based on the *Thyanta* pheromone were prepared with methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate, designated as "*EZZ*," by placing ten rubber septa (West Pharmaceutical Services, Kearney, NE, USA) in a 100-ml, round-bottom, one-neck flask, and covering the septa with an 8-ml hexane solution containing 40 mg of methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate plus 40 mg of zingiberene. The flask was rotated on a rotary evaporator (without applying a vacuum) for 1.5–2 hr or until the liquid was almost completely absorbed into the septa. One set of septa was placed directly into the bottom of traps unprotected from sunlight; this treatment is defined as "U-*EZZ*+Z." Methyl 2,4,6-decatrienoates undergo photoisomerization when exposed to sunlight (Khrimian, unpublished data); therefore, another set of septa was deployed in traps protected from light by pinning the septa inside ~7-cm-long pieces of standard PVC plumbing pipe (2.5 cm ID); this treatment is coded as "*EZZ*+Z." Analogously, a treatment consisting of methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate (4.4 mg/septum) without zingiberene was prepared (designated "*EZZ*"), and septa impregnated with only hexane served as controls (C).

Lures for a second experiment (Exp. 2) were prepared as described above for the *Thyanta*-based treatments except that a second methyl decatrienoate isomer (the *Z*,*E*,*Z*-isomer) was also used, and the compound previously identified as the main pheromone component of *Euschistus* spp. (Aldrich et al., 1991), methyl (*E*,*Z*)-2,4-decadienoate ("*D*"), was used instead of zingiberene. None of these lures was protected from light. Treatments for Exp. 2 consisted of *EZZ*, *EZZ*+*D*, *D*, *ZEZ*, *EZZ*+*D*, and C, with four replicates per treatment.

On September 10, 2004, three additional treatments (four replicates each) were added to Exp. 2 to test the possibility of an interaction between methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate and the male-specific compounds previously identified from the green stink bug, *Acrosternum hilare* (Aldrich et al., 1989; McBrien et al., 2001): (*Z*)-α-bisabolene and (4*S*)-*cis*- and (4*S*)-*trans*-(*Z*)-α-bisabolene epoxides. The commercial sample of bisabolene was determined to contain ~20% (*Z*)-α-bisabolene; therefore, septa were loaded with 20 mg of bisabolene so as to contain 4 mg of the desired isomer for one of the additional treatments ("B"), with a second treatment consisting of *ZEZ* (4 mg) + B (10 mg). The third additional treatment consisted of a 1:1 mixture of (4*S*)-*cis*- and (4*S*)-*trans*-(*Z*)-α-bisabolene epoxides ("X"). The amount of epoxides available was limiting (~12 mg); therefore, only one treatment included the epoxides: *ZEZ* (4 mg) + X (3 mg).

Lures for a third experiment (Exp. 3) were prepared by using rubber septa (four replicates per treatment) impregnated with chemicals as described above, except that each septum was loaded with 2-mg active ingredient/lure. Lures were pinned inside PVC pipe as in Exp. 1 and hung from the roof of sticky traps. Treatments consisted of hexane controls (C), methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate (*EEZ*), methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate (*ZEZ*), and *EEZ*+*ZEZ* (3:1 ratio).

A fourth experiment (Exp. 4) was conducted in 2005 as a follow-up to Exp. 2. Lures were prepared as for Exps. 1 and 2, but methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate was used in place of the (*E*,*Z*,*Z*)-isomer that was part of the Exp. 2 treatment set. Thus, treatments for Exp. 4 consisted of *EEZ*, *EEZ*+*D*, *D*, *ZEZ*, *ZEZ*+*D*, and C, with four replicates per treatment. All treatments for Exp. 4 were protected from light inside PVC pipe as in Exps. 1 and 3. The lures and conditions for the four field trapping experiments are summarized in Table 1.

Table 1 Chemical treatments tested

Exp.	Code	Compound(s)	Formulation	Trap
1	*E*-2H+α-T	(*E*)-2-hexenal +α-terpineol	PVC	Aldrich[a]
1	EZZ_a	Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate[b]	Septum/protected[c]	Aldrich
1	EZZ_a+*Z*	Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate + zingiberene	Septum/protected	Aldrich
1	EZZ_b+*Z*	Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate + zingiberene[d]	Septum/protected	Aldrich
1	U-EZZ_a	Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate	Septum/unprotected[e]	Aldrich
2	U-*EZZ*[f]	Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate	Septum/unprotected	Sterling[g]
2	U-*D*	Methyl (*E*,*Z*)-2,4-decadienoate	Septum/unprotected	Sterling
2	U-*EZZ*+*D*	Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate + methyl (*E*,*Z*)-2,4-decadienoate	Septum/unprotected	Sterling
2	U-*ZEZ*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate	Septum/unprotected	Sterling
2	U-*ZEZ*+*D*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate + methyl (*E*,*Z*)-2,4-decadienoate	Septum/unprotected	Sterling
2	U-*ZEZ*+*B*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate + α-bisabolene	Septum/unprotected	Sterling
2	U-*X*	Bisabolene epoxide	Septum/unprotected	Sterling
2	U-*ZEZ*+*X*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate + bisabolene epoxide	Septum/unprotected	Sterling
3	*EEZ*	Methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate	Septum[h]	Sticky[I]
3	*ZEZ*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate	Septum	Sticky
3	*EEZ*+*ZEZ*	Methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate + methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate	Septum	Sticky
4	*EEZ*	Methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate	Septum/protected	Sterling
4	*D*	Methyl (*E*,*Z*)-2,4-decadienoate	Septum/protected	Sterling
4	*EEZ*+*D*	Methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate + methyl (*E*,*Z*)-2,4-decadienoate	Septum/protected	Sterling
4	*ZEZ*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate	Septum/protected	Sterling
4	*ZEZ*+*D*	Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate + methyl (*E*,*Z*)-2,4-decadienoate	Septum/protected	Sterling

[a] Custom-made clear plastic funnel trap; see text.

[b] First synthetic batch.

[c] Lure pinned inside PVC pipe to shield against sunlight.

[d] Second synthetic batch of methyl (*E,Z,Z*)-2,4,6-decatrienoate.

[e] Unprotected lures (U) were impregnated rubber septa placed in trap bottoms.

[f] Distinction between synthetic batches of methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate was dropped; see text.

[g] Clear plastic funnel trap constructed and supplied by Sterling International.

[h] Lure shielded from direct sunlight by pinning inside trap roof.

[i] Jackson delta traps with removable sticky inserts (Agrisense).

Field Trapping Field experiments were carried out at the USDA Beltsville Agricultural Research Center (BARC), Prince George's County, MD, from June through October of 2004 and August through October of 2005. Experiment 1 was conducted at the South Farm of BARC from June 15 through September 17, 2004, with clear plastic funnel traps designed to capture insects alive (Aldrich et al., 1984a). These traps were hung about 1.8 m above ground from the branches of trees (>20 m apart) in a mixed deciduous forest bordering agricultural fields. Experiment 2 was conducted at the North Farm of BARC from July 1 through October 26, 2004, with traps similar to those used for Exp. 1 supplied by Sterling International (Spokane, WA, USA) and deployed as for Exp. 1. Experiment 3 was conducted from July 9 through October 15, 2004, with Jackson delta traps with

removable sticky inserts (Agrisense, Fresno, CA, USA) (Zhang and Aldrich, 2004) deployed along East Line Road at BARC. Experiment 4 was conducted from August 11 through October 19, 2005, with traps located and positioned as in Exp. 2. Traps with the H+α-T treatment were rebaited every 2–3 d; traps with the other treatments on rubber septa were rebaited every 2 wk. Usually traps were monitored daily; insects caught inside, on, and within ~1 m of a trap were counted. Field-collected insects were preserved for species identification and used for further laboratory experimentation.

Statistical Analysis The total numbers of insects responding to the treatments were analyzed with a Fisher's exact Chi-square test by using StatXact (Mehta and Patel, 2003) to determine if the distribution of the insects differed for the chemical compounds. For each species, the treatment counts were analyzed as a multinomial distribution with StatXact. The probabilities that an individual of the particular species would be attracted to the chemical compound were calculated. Confidence intervals were also calculated for the probabilities. If the confidence intervals overlapped for two treatments, then they were not statistically different. The confidence intervals were calculated by using Sidak adjusted *P* values so that the experiment-wise error was 0.05.

Results

Experiment 1 Although treatments containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate with or without zingiberene were deployed with the expectation of attracting *Thyanta* spp. pentatomids (McBrien et al., 2002b) and their parasitoids, only six individuals of *T. custator accerra* McAtee [one male and five females (1M:5F)] were captured. In fact, three other stink bug species were more abundantly encountered in or around these traps than was *T. custator accerra* (Fig. 2): *A. hilare* (Say) (5M:11F), *Euschistus tristigmus* (Say) (2M:9F), and *Banasa dimidiata* (Say) (5M:3F). Other pentatomids that were caught included *Euschistus servus* (Say) (2F), *P. maculiventris* (Say) (1M:1F), and *Banasa calva* (Say) (1F). No stink bugs were caught in control traps in Exp. 1.

Five species of tachinid flies were captured in or on traps baited with chemical treatments in Exp. 1: *E. flava* (Townsend), *Gymnosoma par* (Walker), *Euthera tentatrix* Loew, *Hemyda aurata* Robineau–Desvoidy, and *Cylindromyia fumipennis* (Bigot) (Fig. 3).

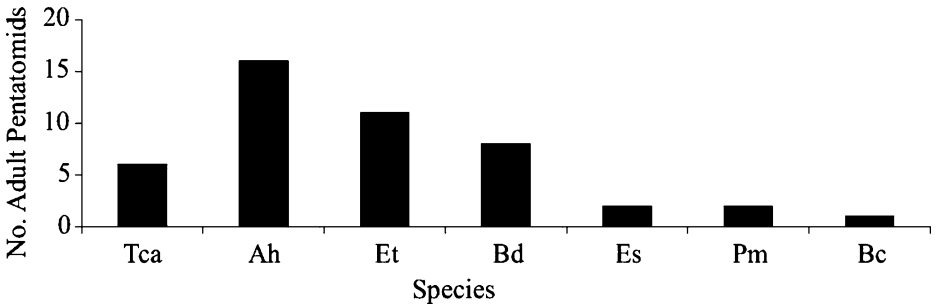


Fig. 2 Adult pentatomid species caught in or around traps baited with lures containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate from June 15 through September 17, 2004 (Exp. 1): *Tca*, *T. custator accerra* McAtee; *Ah*, *A. hilare* (Say); *Et*, *E. tristigmus* (Say); *Bd*, *B. dimidiata* (Say); *Es*, *E. servus* (Say); *Pm*, *P. maculiventris* (Say); and *Bc*, *B. calva* (Say)

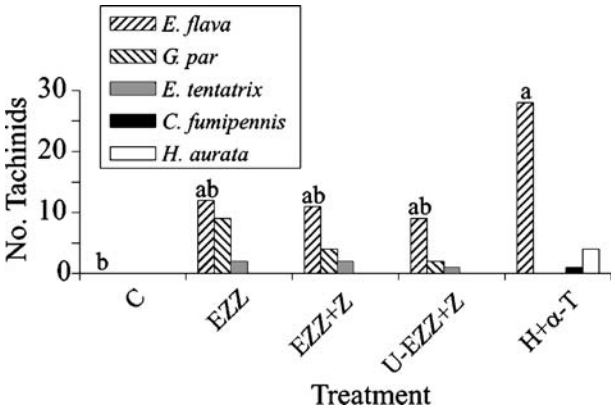


Fig. 3 Tachinid flies caught in or around traps baited with lures containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate (*EZZ*) with or without zingiberene (*Z*) [components of *Thyanta* spp. pheromones (McBrien et al., 2002b)], and (*E*)-2-hexenal with α-terpineol [components of the *P. maculiventris* pheromone, H+α-T (Aldrich et al., 1984a)], from June 15 through September 17, 2004 (Exp. 1): *E. flava* (Townsend), *G. par* (Walker), *E. tentatrix* Loew, *H. aurata* Robineau–Desvoidy, and *C. fumipennis* (Bigot). Bars for *E. flava* with different letters are significantly different ($P<0.05$) (trienoate treatments with the prefix "*U*" were unprotected from light; those without a prefix were protected from light as described in the text)

Two of these tachinid parasitoids (*H. aurata* and *C. fumipennis*) were restricted to traps baited with the major pheromone components of the spined soldier bug, *P. maculiventris*, and two other species (*G. par* and *E. tentatrix*) were restricted to traps with treatments containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate. The most abundantly encountered species was *E. flava*, and this fly was captured in and around traps containing either methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate or the *P. maculiventris* synthetic pheromone. In the former treatments, the presence or absence of zingiberene did not significantly affect attraction of *E. flava*. No tachinids were caught in or around control traps.

In addition to pentatomids and tachinids, one species of solitary wasp was also attracted in Exp. 1 to treatments containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate: *Astata occidentalis* Cresson (Hymenoptera: Sphecidae). A total of seven females were caught inside traps (none in controls) and, as with *E. flava*, the presence of zingiberene appeared not to affect attraction.

Experiment 2 This experiment was designed to further test methyl 2,4,6-decatrienoates by themselves or combined with methyl (*E*,*Z*)-2,4-decadienoate, the previously identified male-specific pheromone component of various *Euschistus* spp. (Aldrich et al., 1991), for attraction of native stink bugs. Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate was included as an extension of Exp. 1 and methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate was included based on preliminary gas chromatography–electroantennogram detector data with tachinid flies (Aldrich et al., 2006) and *H. halys* (Zhang and Aldrich, unpublished data).

Substantial numbers of the dusky stink bug, *E. tristigmus*, and the green stink bug, *A. hilare*, were caught during Exp. 2 at chemically treated traps, but none were caught in or around control traps (Fig. 4). For *A. hilare*, almost 38% of the individuals that were attracted were second- to fifth-instar nymphs, and nymphs were often caught in the absence of adults. Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate alone was significantly attractive to *A. hilare*, whereas methyl (*E*,*Z*)-2,4-decadienoate alone was not attractive to green stink bugs (Fig. 4a). Methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate alone was more attractive to green stink bugs

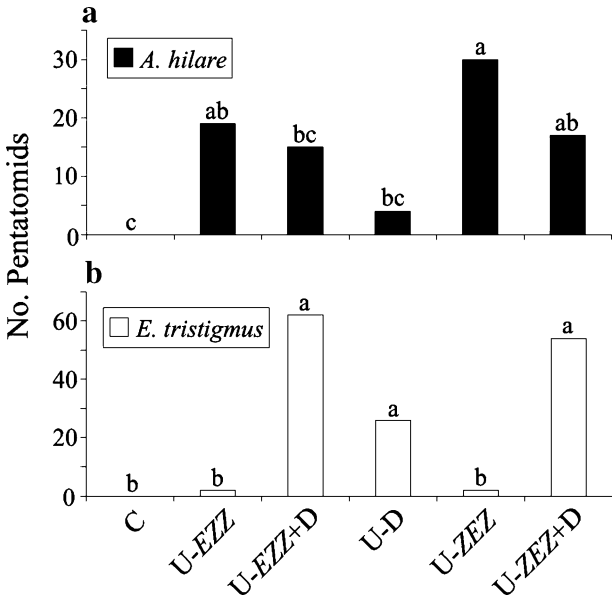


Fig. 4 Total number of pentatomids (adults and nymphs) of **a** the green stink bug, *A. hilare* (Say), and **b** the dusky stink bug, *E. tristigmus* (Say), caught in or around traps baited with lures containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate (*EZZ*), methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate (*ZEZ*), methyl (*E*,*Z*)-2,4-decadienoate (*D*), or the binary combinations *EZZ*+D or *ZEZ*+D from July 1 through October 26, 2004 (Exp. 2). The prefix "*U*" indicates that the lures were unprotected from light. Bars for a species with different letters are significantly different ($P<0.05$)

than control traps, but the number of bugs caught with this decatrienoate isomer was not significantly greater than the number of *A. hilare* captured in and around traps baited with methyl (*E*,*Z*)-2,4-decadienoate alone. Combining either the methyl (*Z*,*E*,*Z*)- or (*E*,*Z*,*Z*)-2,4,6-decatrienoates with methyl (*E*,*Z*)-2,4-decadienoate did not increase attraction over that of the methyl decatrienoates alone (Fig. 4a). More female (33) than male (20) green stink bugs were captured, but this difference was not significant ($X^2=3.19$; $P_{0.05}=3.84$, 1 *df*). No green stink bugs were captured in or around control traps.

For the dusky stink bug, predominantly adults were attracted, with nymphs comprising only about 5% of the *E. tristigmus* individuals caught. Methyl (*E*,*Z*,*Z*)- and (*Z*,*E*,*Z*)-2,4,6-decatrienoates alone were insignificantly attractive to *E. tristigmus*, and the previously known pheromone component [methyl (*E*,*Z*)-2,4-decadienoate] by itself appeared only moderately attractive (Fig. 4b); however, the combination of either methyl (*E*,*Z*,*Z*)- or (*Z*,*E*,*Z*)-2,4,6-decatrienoate with methyl (*E*,*Z*)-2,4-decadienoate did not attract significantly more individuals of *E. tristigmus* than did traps baited with methyl (*E*,*Z*)-2,4-decadienoate alone (Fig. 4b). More *E. tristigmus* females (83) than males (56) were captured ($X^2=5.24$, $P<0.05$). No *E. tristigmus* were caught at control traps.

In Exp. 2, significant numbers of two tachinid species were caught from July 1 through October 26 in or around traps baited with treatments containing methyl (*E*,*Z*,*Z*)- and (*Z*,*E*,*Z*)-2,4,6-decatrienoates, with or without methyl (*E*,*Z*)-2,4-decadienoate: *E. flava* and *G. par* (Fig. 5). *Euclytia flava* was attracted to methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate, but methyl (*E*,*Z*)-2,4-decadienoate was completely unattractive to this fly (Fig. 5a). Methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate was not attractive to *E. flava* in this experiment, and combining methyl (*E*,*Z*)-2,4-decadienoate with methyl (*E*,*Z*,*Z*)- and (*Z*,*E*,*Z*)-2,4,6-decatrienoate did not increase attraction of *E. flava* over that of these decatrienoates alone. For *G. par* (Fig. 5b), only treatments containing methyl (*E*,*Z*)-2,4-decadienoate were attractive, and the combinations of either methyl (*E*,*Z*,*Z*)- or (*Z*,*E*,*Z*)-2,4,6-decatrienoates with methyl (*E*,*Z*)-2,4-decadienoate were not more attractive than the decadienoate alone. Single specimens of *E. tentatrix* were caught with each chemical treatment, but no tachinids were caught at control traps.

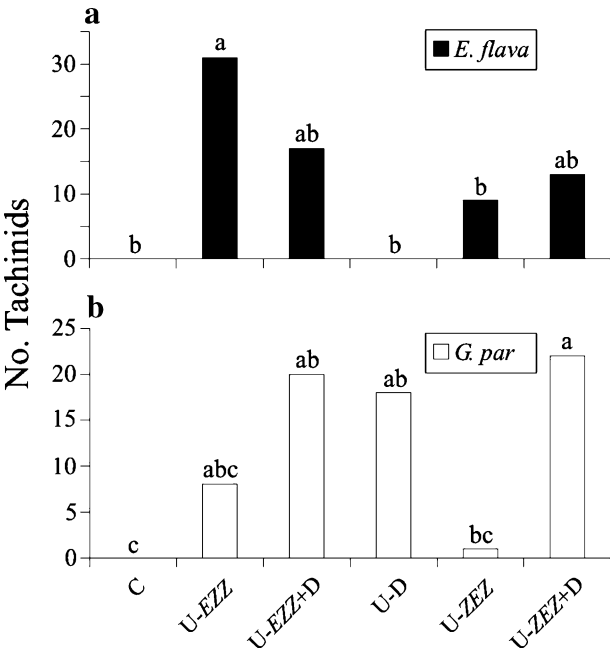


Fig. 5 The tachinid flies, **a** *E. flava* (Townsend) and **b** *G. par* (Walker), caught in or around traps baited with lures containing methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate (*EZZ*), methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate (*ZEZ*), methyl (*E*,*Z*)-2,4-decadienoate (*D*), or the binary combinations *EZZ*+D or *ZEZ*+D from July 1 through October 26, 2004 (Exp. 2). The prefix "*U*" indicates that the lures were unprotected from light. Bars for a species with different letters are significantly different ($P<0.05$)

Figure 6 shows the numbers of pentatomids (Fig. 6a) and tachinids (Fig. 6b) caught in or around traps for Exp. 2 from September 10 through October 26, 2004, when treatments were added containing (*Z*)-α-bisabolene or (4*S*)-*cis*- and (4*S*)-*trans*-(*Z*)-α-bisabolene epoxides. Treatments containing (*Z*)-α-bisabolene or (*Z*)-α-bisabolene epoxides did not significantly attract either *E. tristigmus* or *A. hilare* (Fig. 6a). Among the tachinids, *Trichopoda pennipes* (F.) was encountered for the first time, but only in the treatment containing methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate with (4*S*)-*cis*- and (4*S*)-*trans*-(*Z*)-α-bisabolene epoxides (Fig. 6b); 12 individuals of this species (six males and six females) were caught.

Experiment 3 This experiment was conducted to determine if there are native tachinids preadapted to recognize the two methyl decatrienoate isomers thought most likely to attract *H. halys*; i.e., methyl (*E*,*E*,*Z*)- and (*Z*,*E*,*Z*)-2,4,6-decatrienoates. The isomer identified from *P. stali* (Sugie et al., 1996) that is reportedly cross-attractive to *H. halys* in Japan [methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate] (Tada et al., 2001a, b) is, in fact, highly attractive to females of *G. par* (Fig. 7). However, the (*Z*,*E*,*Z*)-isomer was totally unattractive to *G. par*.

Experiment 4 As in Exp. 2, for *A. hilare*, a substantial number of the attracted individuals were nymphs, about 16% of the 127 green stink bugs that were caught. Methyl (*Z*,*E*,*Z*)- and (*E*,*E*,*Z*)-2,4,6-decatrienoate alone were significantly attractive to *A. hilare*, whereas methyl (*E*,*Z*)-2,4-decadienoate alone was not attractive to green stink bugs (Fig. 8a). Combining methyl (*E*,*Z*)-2,4-decadienoate with methyl (*Z*,*E*,*Z*)- or (*E*,*E*,*Z*)-2,4,6-decatrienoates did not increase attraction. The number of females captured (50) was not significantly different than the number of males caught (54). No green stink bugs were captured in or around control traps.

For the dusky stink bug, predominantly adults were attracted, with nymphs comprising about 8% of the *E. tristigmus* individuals caught. Methyl (*E*,*E*,*Z*)- and (*Z*,*E*,*Z*)-2,4,6-decatrienoates alone were insignificantly attractive to *E. tristigmus*. The previously known pheromone component [methyl (*E*,*Z*)-2,4-decadienoate] by itself was attractive (Fig. 8b).

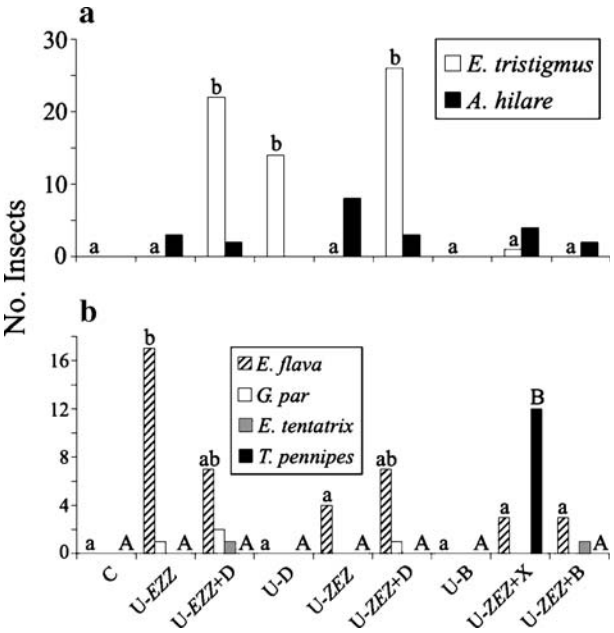


Fig. 6 The total numbers of pentatomids (**a**) and tachinids (**b**) caught in or around traps when treatments containing (*Z*)-α-bisabolene (*B*) or (4*S*)-*cis*- and (4*S*)-*trans*-(*Z*)-α-bisabolene epoxides (*X*) were added to Exp. 2 from September 10 through October 26, 2004 (see text for details). The prefix "*U*" indicates that the lures were unprotected from light. Bars for a species with different letters are significantly different ($P<0.05$)

As in Exp. 2, the addition of methyl 2,4,6-decatrienoates [in this experiment, the (*E*,*E*,*Z*)- and (*Z*,*E*,*Z*)-isomers] to methyl (*E*,*Z*)-2,4-decadienoate did not increase the number of *E. tristigmus* individuals caught relative to traps baited with methyl (*E*,*Z*)-2,4-decadienoate alone (Fig. 8b). In this experiment, the number of *E. tristigmus* females caught (121) was not significantly greater than the number of males captured (108). No *E. tristigmus* were caught at control traps.

Finally, the first captures of the BMSB, *H. halys*, were recorded in Beltsville, MD, during Exp. 4. Three fifth-instar *H. halys* nymphs were caught inside traps baited with methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate on August 15 and 18, and one adult *H. halys* male was captured inside a trap baited with methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate plus methyl (*E*,*Z*)-2,4-decadienoate on September 1, 2005. The cuticle of the adult male was soft and pale in coloration, suggesting it recently eclosed to adult.

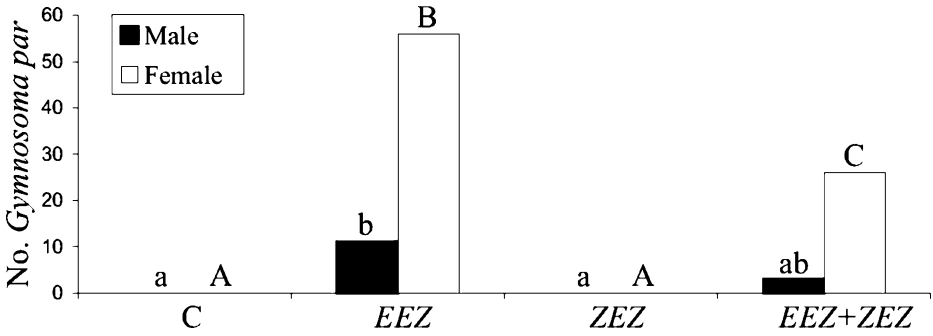


Fig. 7 The total number of *G. par* (Walker) caught in sticky traps baited with methyl (*E*,*E*,*Z*)- and/or (*Z*,*E*,*Z*)-2,4,6-decatrienoate (*EEZ* and *ZEZ*) from July 9 through October 15, 2004. Bars for a species with different letters are significantly different ($P<0.05$)

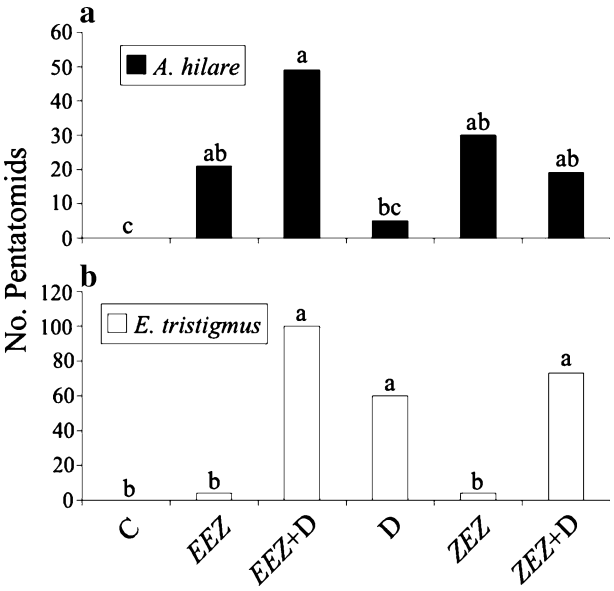


Fig. 8 The total number of pentatomids (adults and nymphs) of **a** the green stink bug, *A. hilare* (Say), and **b** the dusky stink bug, *E. tristigmus* (Say) caught in or around traps baited with lures containing methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate (*EEZ*), methyl (*Z*,*E*,*Z*)-2,4,6-decatrienoate (*ZEZ*), methyl (*E*,*Z*)-2,4-decadienoate (*D*), or the binary combinations *EEZ*+D or *ZEZ*+D from August 11 through October 19, 2005 (Exp. 4). Bars for a species with different letters are significantly different ($P<0.05$)

Discussion

Results presented herein, along with data from field tests conducted near Allentown, PA (Khrimian et al., 2006), verify that the BMSB, *H. halys* (Stål) (Pentatomidae) is, indeed, attracted to methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate (Tada et al., 2001a, b) and related geometric isomers. In addition, tests of synthetic pheromone for *T. custator accerra* McAtee show that the tachinid fly parasitoid, *E. flava* (Townsend), does use the pheromone of *Thyanta* as a host-finding kairomone. However, the most surprising result from our field research is the discovery that other native stink bugs and tachinids are also attracted to methyl 2,4,6-decatrienoates.

Two species, in particular, were significantly attracted to certain methyl 2,4,6-decatrienoates: the green stink bug, *A. hilare* (Say), and the tachinid fly, *G. par* (Walker). Each of these species is native to North America, yet what is known of their chemical ecology raises more questions than answers as to why they are attracted to methyl decatrienoates.

Gymnosoma spp. are known parasitoids of *Euschistus* stink bugs (Arnaud Jr., 1978; Aldrich, 1995) but do not normally parasitize *A. hilare* (see also: Eger and Ables, 1981; Jones et al., 1996). We found that *G. par* females are attracted to methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate, the methyl decatrienoate isomer seemingly most attractive to the BMSB, suggesting to us that this fly might be preadapted to recognize *H. halys* in the USA. In fact, field-collected *G. par* caged in the laboratory with *H. halys* adults did profusely oviposit on the bugs (exclusively underneath the wings) (Aldrich et al., 2006). However, examination of several hundred wild BMSB adults from Allentown failed to reveal any eggs underneath the wings of these bugs. On the other hand, two *T. pennipes* (F.) were reared from field-collected *H. halys* adults (Aldrich et al., 2006); *T. pennipes* is the only known tachinid parasitoid of *A. hilare* in North America (Arnaud Jr., 1978). While *G. par* was highly attracted to methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate, this fly was totally indifferent to the (*Z*,*E*,*Z*)-isomer. The fact that this abundant North American tachinid parasitoid of pentatomids is so

finely tuned to a compound yet to be found from any native heteropteran is probably an indication of our semiochemical ignorance; there must be other bugs in North America that produce methyl decatrienoates as part of their attractant pheromones.

A clue as to which pentatomid species might be producing methyl 2,4,6-decatrienoates as pheromone components comes from prey records of the solitary wasp, *A. occidentalis* Cresson (Sphecidae). Females of this sphecid use methyl (*E*,*Z*,*Z*)-2,4,6-decatrienoate as a kairomone to find *Thyanta* adults with which to provision their nests (Millar et al., 2001, and data presented herein). In addition to *Thyanta* spp., Evans (1957) lists *Hymenarcys nervosa* (Say) (Pentatomidae) as the most abundant prey of *A. occidentalis* in Indiana, and also recorded *B. calva* (Say) (Pentatomidae) as prey. Notably, in our 2004 test of synthetic *T. custator accerra* pheromone, more individuals of *B. dimidiata* (Say) were attracted than *Thyanta*, and one individual of *B. calva* was captured.

Why are nymphs and adults of the green stink bug, *A. hilare*, attracted to methyl 2,4,6-decatrienoates? Male-specific volatiles identified from *A. hilare* include straight-chain hydrocarbons, a cyclic sesquiterpene [(*Z*)-α-bisabolene], and *cis*- and *trans*-(*Z*)-α-bisabolene epoxides (Aldrich et al., 1989, 1993); compounds quite unlike methyl decatrienoates. McBrien et al. (2001) reinvestigated the suspected pheromone chemistry of *A. hilare*, demonstrating that females are significantly attracted to a blend of synthetic (*Z*)-α-bisabolene epoxides mimicking the natural blend, but methyl decatrienoates were not among the volatiles isolated from *A. hilare* in this study either, nor are there records of these types of methyl esters from plants. To our knowledge, the most closely related known phytochemicals are 2,4,6-decatrienoic acids from certain *Euphorbia* spp. (Warnaar, 1977, 1981), plants unlikely to be hosts of any of the stink bugs in question here (McPherson, 1982).

If the only natural sources of methyl decatrienoates are *Thyanta* and some other yet-to-be-discovered species of bugs, then one possible explanation for the observed attraction of *A. hilare* to these esters is that green stink bugs are cross-attracted to the pheromones of these other bugs. How plausible an explanation is this? Certainly the most clear-cut examples of heteropterans exploiting the signals of other species are cases involving predation, such as kairomonal attraction of predacious anthocorid bugs to sex pheromones of pine bast scales (Hemiptera: Matsucoccidae) (Mendel et al., 2004), and chemicals (Yasuda, 2000), or even vibrations (Pfannenstiel et al., 1995), associated with the prey of predacious pentatomids. A convincing case of cross-attraction between heteropterans within the same trophic level, however, has recently been reported by Endo et al. (2006). While investigating a complex of bugs that are pests of soybean in Japan, these authors found that the pentatomid, *Piezodorus hybneri* (Gmelin), is significantly attracted to a pheromone component of a competitor, *Riptortus clavatus* (Thunberg) (Heteroptera: Alydidae). These two species have no pheromone compounds in common, nor do soybean plants produce the cross-attractive compound. Endo et al. (2006) speculated that *P. hybneri* utilizes the aggregation pheromone of *R. clavatus* as a kairomone to search for food plants. In fact, the reported cross-attraction of *H. halys* to the pheromone of *P. stali* [i.e., methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate] (Tada et al., 2001a, b) may be another example of such a kairomone.

Acrosternum hilare has a distinct preference for certain native hosts, mainly wild bushes and trees, and a succession of hosts appears to be necessary to sustain a population (Schoene, 1933). Cultivated crops are usually damaged only when the wild-host succession is broken. Given this phenology, it may be adaptive for green stink bug adults and nymphs to go to the pheromones of congeneric bugs in the habitat. The fact that pentatomid nymphs, even young nymphs, are often attracted to the pheromones of conspecific males (Aldrich, 1988a, 1995) supports the idea that pheromones may be associated with food because nymphs are obviously not seeking a mate.

Aside from finding food or a mate, another possible advantage of gregariousness for animals is defense, due to the so-called selfish herd effect, whereby the probability of an individual being attacked by a predator decreases (is diluted) the larger the group (Hamilton, 1971). The concept is relevant not just in cases of predation but also as a passive means of defense against parasites (Mooring and Hart, 1992) and parasitoids (Wcislo, 1984) and applies to mixed species aggregations as well (Hamilton, 1971; Watt and Chapman, 1998; Gibson et al., 2002; Pereira et al., 2004).

The extent to which tachinid parasitoids cue on heteropteran semiochemicals to find these hosts is striking (Aldrich, 1995; Aldrich and Zhang, 2002; Aldrich et al., 2006). Ironically, the stink gland secretions of stink bugs are, by and large, ineffective against tachinids, and the allomones of bugs are even occasionally usurped as kairomones by these flies (Aldrich, 1995). Early tests of the aggregation pheromone for the predatory spined soldier bug, *P. maculiventris* (Say) (Pentatomidae), demonstrated that the bugs avoid entering traps on which tachinid parasitoids are perched (Aldrich et al., 1984a). Thus, tachinids probably have been and continue to be a major selective agent of Heteroptera. Most tachinid parasitoids of Heteroptera belong to the subfamily Phasiinae, which lay a single macrotype egg on a host with only one tachinid larva able to develop per host. Phasiines do not scent-mark their hosts and seem unable to recognize the presence of other eggs on a bug (Stireman et al., 2006); therefore, when these flies are confined in cages with potential hosts, they usually oviposit repeatedly on the same bugs (Aldrich et al., 2006). It is unlikely that this is how phasiines behave in nature. One can imagine that these flies search for a potential host, oviposit on one host whether it is part of an aggregation or solitary, and then fly off to find another host patch. If this scenario is accurate, then the common gregarious behavior of bugs may represent a selfish herd passive defense against tachinid parasitism. Interestingly, mixed aggregations of *H. halys* and *A. hilare* nymphs have occasionally been seen in the field near Allentown, PA (Gary Bernon, USDA-APHIS, personal communication).

Despite the mystery of why various heteropterans and their parasitoids are attracted to methyl decatrienoates, the discovery that these compounds are attractive to economically important bugs may provide another useful semiochemical tool for monitoring and management (e.g., Cullen and Zalom, 2005). This is especially important now that there is a resurgence of problems with bugs because of their immunity to the Bt endotoxin in genetically modified crops (Millar et al., 2002).

Acknowledgements We thank T. J. Henry, Systematic Entomology Laboratory (SEL), USDA-ARS, Beltsville, MD, and David Rider, North Dakota State University, Fargo, for identification of pentatomid species, and Norman Woodley, SEL, for identification of tachinids. We are also grateful to Peter Shearer, Rutgers University, Agricultural Research & Extension Center, Bridgeton, NJ, for supplying *H. halys*, and to John Reese, Department of Entomology, Kansas State University, Manhattan, for helpful discussions and assistance with field work.

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J Chem Ecol (2007) 33:817–823
DOI 10.1007/s10886-007-9267-4

RAPID COMMUNICATION

Acetophenone as an Anti-attractant for the Western Pine Beetle, *Dendroctonus Brevicomis* LeConte (Coleoptera: Scolytidae)

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Received: 26 September 2006 / Accepted: 15 February 2007 /
Published online: 22 February 2007
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Abstract Host location and colonization by bark beetles is dependent upon the relative and absolute amounts of attractant and antiattractant compounds available. Many investigations have lead to use of antiattractants for the management of these pests and have been especially focused on verbenone. However, recent studies have identified new anti-attractants for several species of bark beetles. We report results of recent investigations of the response of western pine beetle, *Dendroctonus brevicomis* LeConte, to two recently identified antiattractants, acetophenone, and fenchyl alcohol, with verbenone as a standard of comparison, in northern California. Release of both acetophenone and verbenone resulted in significantly lower trap catches of *D. brevicomis* in aggregation pheromone-baited traps, while fenchyl alcohol was inactive. Acetophenone was the only antiattractant that did not reduce numbers of the most abundant predator of *D. brevicomis*, *Temnochila chlorodia* (Mannerheim), responding to the attractant pheromone of its prey. Aggregation pheromone-baited traps with acetophenone also had the highest predator/prey ratio. Our results suggest that acetophenone may be part of the intra- and interspecific interactions among sympatric species of bark beetles and may have application in their control.

Keywords Acetophenone · Verbenone · Fenchyl alcohol · Antiattractants · Bark beetles · Predators

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Introduction

The western pine beetle (WPB), *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae), is indigenous to western conifer forests. Its hosts are ponderosa pine, *Pinus ponderosae* Lawson, and Coulter pine, *Pinus coulteri* D. Don. In order for WPB progeny to survive, the parent beetle population must kill the tree, so large numbers of beetles must aggregate in synchrony on a single host tree. Many species of bark beetles release aggregation pheromones that attract both mates and additional colonizers, thus enhancing their tree killing ability (Wood 1982). WPB releases *exo*-brevocomin ((+)-*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1] octane), (−)-frontalin ((−)-1,5-dimethyl−6,8-dioxabicyclo [3.2.1]octane), and a host terpene, myrcene, which attracts large numbers of conspecifics during the aggregation phase of host colonization. In addition, some bark beetles, including WPB, produce antiaggregation pheromones that inhibit attraction of conspecifics to aggregation pheromones to optimize resource partitioning (Paine and Hanlon 1991).

Many investigations have led to the use of antiaggregation pheromones, such as verbenone, to prevent or minimize damage caused by beetle infestation (Borden 1996). Verbenone, an antiaggregation pheromone of several bark beetle species, was the focus of earlier studies with WPB (Paine and Hanlon 1991). Earlier studies investigated the inhibitory effect of verbenone in combination with semiochemicals produced by heterospecific bark beetles (Paine and Hanlon 1991; Bertram and Paine 1994a,b). Tests of verbenone to interrupt host colonization by WPB, in some cases, reduced trap catch, but in others were inconsistent in protecting individual trees (Paine and Hanlon 1991; Bertram and Paine 1994a,b).

Recent investigations identified previously unknown semiochemicals for several species of bark beetles (Pureswaran and Borden 2004; Sullivan 2005). Some of these semiochemicals are potential inhibitors of bark beetle attraction (Pureswaran and Borden 2004; Sullivan 2005). In the current study, we tested two of these recently identified antiattractant compounds—acetophenone and fenchyl alcohol. Our objective was to compare the inhibition of WPB response by these compounds relative to verbenone to its aggregation pheromone.

Methods and Materials

(*1S*)-(−)-Verbenone (chemical purity: ≥93%, enantiomeric purity ~97%) and two antiattractant compounds, acetophenone (chemical purity: ≥98%) and (*1R*)-(+)-fenchyl alcohol (chemical purity: ≥97%, enantiomeric purity ~97%), each combined with the aggregation pheromone of WPB, as well as WPB aggregation pheromone alone, were presented to WPB in a replicated test in a ponderosa pine forest managed by Hancock Forest Management, Siskyou County in northern California. All chemicals except for the aggregation pheromone were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The aggregation pheromone (racemic, chemical purity: >97%) was obtained from Phero Tech International, Inc. (Delta, British Columbia, Canada). All antiattractant compounds were released from 15-ml polyethylene bottles (Synergy Semiochemicals, Burnaby, British Columbia, Canada), each filled with 8 ml of one compound. The aggregation pheromone was released from membranes with bubble cap reservoirs. To ensure that the entire contents of each vial were exposed to the release surface, the bottles were squeezed until the liquid reached the shoulder, and then were tightly capped leaving the bottles slightly collapsed. Each bottle also contained 100 mg of the antioxidant BHT

(butylhydroxytoluene) (Sigma-Aldrich Chemical Co.). Approximate release rates of verbenone, fenchyl alcohol, and acetophenone were 2.5, 4.9, and 7.25 mg/d, respectively, at 15.1±4.3°C with a wind velocity of 16.2 km/hr±5.3 for at least 30 d in the field. The release rates of chemicals were determined by daily weight loss under the same conditions. The release rates of different compounds of WPB pheromone, frontalin, *exo*-brevicomin, and myrcene are 3.0, 3.0, and 18.0 mg/d at 24°C, respectively (data provided by Phero Tech International).

The experimental design consisted of six replications/treatment (six traps), completely randomized with repeated measurements of one data collection per week during 5 wk (30 counts/treatment samples for pheromone or pheromone with acetophenone; 29 counts for pheromone with verbenone or pheromone with fenchyl alcohol due to missing data for one trap on the third and second collection period, respectively); in some sampling occasions, the traps were rotated between sites. Release devices were suspended in the middle of flight intercept traps (Advanced Pheromone Technologies, Marylhurst, OR, USA). Traps were suspended from metal standards more than 2 m above ground and spaced at least 15 m from any other trap. An insecticidal strip (Hercon Environmental Co., Emigsville, PA, USA) was placed in each collection cup to prevent predation. Trapped beetles were collected every 7 d from June 21 to July 27, 2006. Beetles captured in each trap were identified, counted, and recorded; voucher specimens were stored at the USDA Forest Service Institute of Forest Genetics, Placerville, CA, USA, and the University of California, Berkeley.

Statistical Analyses Mean numbers of beetles per trap per sampling intervals (week) were analyzed with Poisson Regression Models for overdispersed Poisson distributed responses (counts) to address potential overdispersion arising from the repeated measurements (McCulloch and Searle 2001). This Poisson Regression model belongs to the family of the Mixed Generalized Linear Model (G LM). Model:

$$\text{Expected}\left[\text{count}_{i,j,\text{site}}|\varepsilon_{\text{site}}\right] = e^{\text{Treat}*\text{week}_{ij}+\varepsilon_{\text{site}}}$$

Where i=1,2,3,4 (four treatments) and j=1,2,3,4,5 (five weekly periods), ε is the overdispersion error due to the same site being used for measurements in different weeks (Julian days), "|" means "conditioned to", and "*" means "interaction".

The predator/prey ratio was estimated by using a similar model for the predator counts with the logarithm of number of prey as an offset:

$$\text{Expected}\left[\text{Predator count}_{i,j,\text{site}}|\varepsilon_{\text{site}}\right] = e^{\text{Treat}_i+\text{week}_j+\log\left(\text{Prey count}_{i,j,\text{site}}\right)+\varepsilon_{\text{site}}}$$

The generalized estimating equations (Liang and Zeger 1986) method was used to estimate the model's parameters with SAS (v. 9.1.3) GENMOD procedure, and the Wald chi-square test with the Bonferroni adjustment was used for multiple comparisons for an experiment-wise error rate of 0.05. The data per trap are not independent because the same site is measured during the five occasions (five sampling periods). The statistical analysis accounted for these repeated measurements. The analysis was done per week as is shown in the statistical model above. The counts for the whole period per trap were obtained by summing the estimated weekly counts.

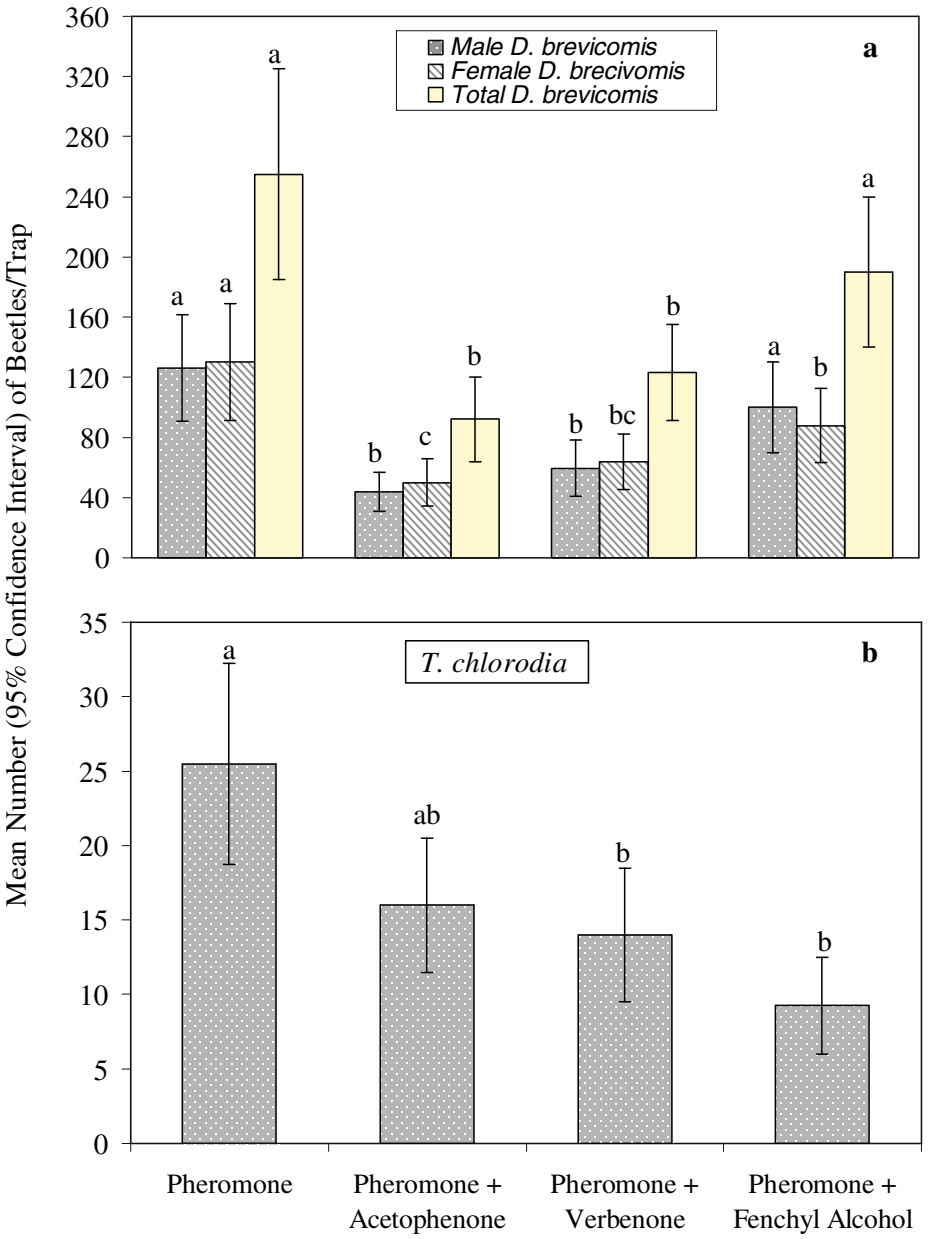


Fig. 1 Field responses of *Dendroctonus brevicomis* and *Temnochila chlorodia* to flight intercept traps baited with aggregation pheromone (P) of *D. brevicomis* alone or with acetophenone (A), verbenone (V), or fenchyl alcohol (F) in a ponderosa pine forest in Northern California. Data show mean numbers (95% lower and upper confidence interval) of beetles per trap summed over the sampling period (five sampling periods) for each treatment level from June 28 to July 27. There were a total of six traps/treatment (30 counts for P or P+A, 29 counts for P+V or P+F due to missing data for one trap on the third and second collection period, respectively). Means followed by the same letter are not significantly different from each other (P − values $< 0.05/6 = 0.008$, Bonferroni adjusted). The weekly mean counts per trap were estimated assuming the Generalized Linear Models (GLM) for overdispersed Poisson counts. **(a)** Mean numbers of males and females and total numbers of *D. brevicomis*. Note that intervals can overlap and still the treatment level can be significant as for the case F versus V. **(b)** Mean numbers of *T. chlorodia*

Results and Discussion

A total of 4,461 beetles representing two species were caught over the experimental period. The WPB was 91.3% of total catch, and the remaining 8.7% was the predator *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae). The overall male/female ratio of *D. brevicomis* was 1.008.

We found significant treatment effects in the response of both WPB and *T. chlorodia* ($P-$ values $< 0.05/6 = 0.008$, Bonferroni adjusted). Both acetophenone and verbenone reduced the catch of WPB attracted to its aggregation pheromone, whereas fenchyl alcohol did not (Fig. 1a). Although the mean number of WPB caught in traps baited with the aggregation pheromone and acetophenone was about 30% lower than that in traps baited with the aggregation pheromone and verbenone, this difference was not significant. The sex ratio of WPB trapped by the aggregation pheromone was not altered by combination with any antiattractant compound except fenchyl alcohol (Fig. 1a), which significantly reduced the relative proportion of females trapped by the aggregation pheromone alone (M:F= 1.15:1).

Inhibition of the response of WPB to its aggregation pheromone by acetophenone is the first report for this species. Acetophenone has been shown to inhibit attraction of the southern pine beetle, *Dendroctonus frontalis* Zimmermann, and the female Douglas-fir beetle, *Dendroctonuspseudotsugae* Hopkins, to their aggregation pheromones in the field (Pureswaran and Borden 2004; Sullivan 2005).

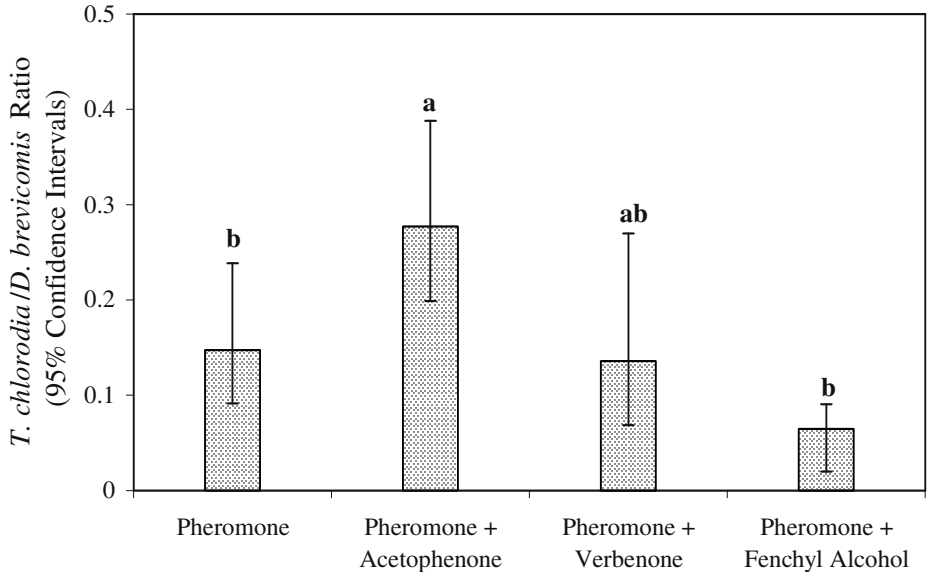


Fig. 2 Estimated mean and 95% confidence intervals of *T. chlorodia*/*D. brevicomis* ratio to flight intercept traps baited with aggregation pheromone (P) of *D. brevicomis* alone or with acetophenone (A), verbenone (V), or fenchyl alcohol (F) in a ponderosa pine forest in Northern California. Data show mean numbers (95% lower and upper confidence interval) of beetles per trap summed over the sampling period (five sampling periods) for each treatment level from June 28 to July 27. There were a total of six traps/treatment (30 counts for P or P+A, 29 counts for P+V or P+F due to missing data for one trap on the third and second collection period, respectively). Means followed by the same letter are not significantly different from each other ($P-$ values $< 0.05/6 = 0.008$, Bonferroni adjusted)

We do not know if WPB produces acetophenone, but it has been identified in volatiles from female *D. pseudotsugae* Hopkins, *Dendroctonus rufipennis* (Kirby), and *Dryocoetes confuses* Swaine; male *Taphrorychus bicolor* (Herbst); and both male and female *D. ponderosae* and *Ips pini* (Say) (references in Pureswaran et al. 2000). The inhibitory effect of acetophenone on WPB attraction suggests that either it is a part of the antiaggregation pheromone of WPB, as suggested for *D. ponderosae* (Pureswaran et al. 2000), or it is used to avoid competition with other bark beetle species. *Ips paraconfusus* Lanier, *I. Pini*, and *D. ponderosae* compete with WPB in the same host trees. Both *I. pini* and *D. ponderosae* produce and respond to acetophenone (Pureswaran et al. 2000). It has also been demonstrated that pheromones of competitors of WPB reduced trap catches of WPB responding to its pheromone (Wood 1982; Paine and Hanlon 1991; Bertram and Paine 1994a,b). Hence, production of acetophenone combined with aggregation pheromones of coinhabiting species may indicate unsuitable hosts for further colonization (Bertram and Paine 1994a,b; Pureswaran et al. 2000).

Catches of *T. chlorodia* were also altered by addition of antiattractants to the aggregation pheromone of WPB (Fig. 1b). Acetophenone was the only antiattractant that did not reduce numbers of *T. chlorodia* responding to the attractant pheromone of its prey. Moreover, the *T. chlorodia*/WPB ratio was highest for traps with acetophenone (0.28), which was 1.9 times higher than with the aggregation pheromone alone (0.15), 2.2 times higher than with verbenone (0.13), and 4.7 times higher than with fenchyl alcohol (0.06) (Fig. 2). Likewise, Sullivan (2005) found no difference in attraction of predators of *D. frontalis* to its pheromone when released with various antiattractants produced by *D. frontalis*, including acetophenone, in southern US. This result has important implication for the pest management of WPB. Although both acetophenone and verbenone reduced the catches of WPB, they did not completely stop WPB from reaching traps baited with its aggregation pheromone. Hence, arrival of higher number of predators may help to reduce WPB population in WPB attacked trees. However, the relationship between trapping the WPB and subsequent tree mortality needs to be evaluated with respect to the impact on predator populations.

For three reasons, our results suggest that acetophenone has promise for pest management strategies against the WPB. First, among the compounds we tested, acetophenone appears to be at least as effective as verbenone in inhibiting the response of WPB. Second, acetophenone was the only compound tested that did not interfere with the attraction of predators, and it produced the highest predator/prey ratio of the four treatments tested. Third, acetophenone is currently much more economical than verbenone. The cost for 1 kg of acetophenone and verbenone are $34 and $400, respectively, as of September, 2006 (Sigma-Aldrich Chemical Co.). This price difference may allow acetophenone to be used for large-scale experiments. Our study is the first step in a series of field bioassays for demonstrating the antiattractant activity of acetophenone for WPB. Before the effectiveness and utility of a potential treatment can be determined, further experiments are needed to determine the threshold ratio of aggregation pheromone to acetophenone and verbenone.

Acknowledgements We thank Jeffrey Webster (Total Forestry, Redding, CA), Fabian Uzoh (USDA Forest Service, Redding, CA), and Emmanuel Uzoh (USDA Forest Service, Redding, CA, volunteer) for valuable assistance with installation and maintenance of the study. We thank James Wolter (Hancock Forest Management) for permission to conduct this study on private lands. The authors are also thankful to three anonymous reviewers and an associate editor (Dr. Monika Hilker) for their help on the previous draft of this manuscript.

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J Chem Ecol (2007) 33:825–838
DOI 10.1007/s10886-007-9265-6

Courtship Pheromones in Parasitic Wasps: Comparison of Bioactive and Inactive Hydrocarbon Profiles by Multivariate Statistical Methods

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Received: 17 July 2006 / Revised: 22 November 2006 / Accepted: 27 November 2006 /
Published online: 1 March 2007
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Abstract Cuticular hydrocarbons play a significant role in the regulation of cuticular permeability and also in the chemical communication of insects. In the parasitoid *Lariophagus distinguendus* (Hymenoptera: Pteromalidae), male courtship behavior is mediated by a female-produced sex pheromone. Previous studies have shown that the chemicals involved are already present in the pupal stage of both males and females. However, pheromonal activity in males decreases shortly after emergence. This pheromonal deactivation occurs only in living males, suggesting an active process rather than simple evaporation of bioactive compounds. Here, we present evidence that the sex pheromone of *L. distinguendus* is composed of a series of cuticular hydrocarbons. Filter paper disks treated with nonpolar fractions of cuticular extracts of freshly emerged males and females, 72-hr-old females, and yellowish pupae caused arrestment and stimulated key elements of courtship behavior in males, whereas fractions of 72-hr-old males did not. Sixty-four hydrocarbons with chain length between C_{25} and C_{37} were identified in the fractions by gas chromatography-mass spectrometry (GC-MS). Methyl-branched alkanes with one to four methyl groups were major components, along with traces of *n*-alkanes and monoalkenes. Principal component analysis, based on the relative amounts of the compounds, revealed that cuticular hydrocarbon composition differed among all five groups. By using partial least squares-discriminant analysis, we determined a series of components that differentiate bioactive and bioinactive hydrocarbon profiles, and may be responsible for pheromonal activity of hydrocarbon fractions in *L. distinguendus*.

Keywords Parasitoid · *Lariophagus distinguendus* · Pteromalidae · Sex pheromone · Cuticular hydrocarbons · Principal component analysis · PCA · Partial least squares-discriminant analysis · PLS-DA

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Introduction

Hydrocarbons are found on the cuticle of almost all insects. In addition to their primary function as a water loss barrier (Nelson and Blomquist, 1995; Gibbs, 1998), cuticular hydrocarbons are known to be involved in semiochemical communication of insects (Howard, 1993; Blomquist et al., 1993, 1998; Howard and Blomquist, 2005). In social insects, cuticular hydrocarbons contribute to the recognition of species, caste, and nestmates (Blomquist et al., 1998), and may facilitate orientation toward the nesting site (Steinmetz et al., 2003). Furthermore, cuticular hydrocarbons are involved in short-range sexual communication of insects, enabling recognition of sexual mates, causing aggregation, or acting as a courtship inhibitor to reduce the attractiveness of mated females (Blomquist et al., 1993; Ferveur, 2005).

Compared to other insect taxa, there are relatively few studies dealing with the composition of cuticular hydrocarbons with respect to their potential role as semiochemicals in parasitic wasps. A number of investigations have focused on comparisons of qualitative and quantitative differences in hydrocarbon profiles of parasitoids with respect to species, sex, or the host used for development (Howard, 1992, 2001; Howard and Infante, 1996). However, few studies have included bioassays demonstrating behavioral activity of synthetic hydrocarbons or hydrocarbon fractions. In *Cardiochiles nigriceps* (Braconidae), elements of male courtship behavior are elicited by a series of female-specific alkadienes (Syvertsen et al., 1995). Recent studies on the pteromalids *Roptrocerus xylophagorum* (Sullivan, 2002), *L. distinguendus* (Steiner et al., 2005), and *Nasonia vitripennis* (Steiner et al., 2006) have demonstrated that nonpolar hydrocarbon fractions from females arrest males and elicit courtship behavior. Interestingly, in *L. distinguendus*, pupae of both sexes also elicited male courtship behavior. However, 32 hr after emergence, males became behaviorally inactive to male conspecifics. To date, it is not known which components contribute to the biological activity of extracts in Pteromalidae.

Cuticular hydrocarbon profiles of insects are generally complex, often consisting of more than a hundred components, mainly saturated and unsaturated (one to three double bonds) straight chain and methyl-branched (one to four branches) alkanes (Lockey, 1988). Studies on social insects have shown that subtle quantitative differences in cuticular hydrocarbon profiles can be detected by analyzing chemical data with multivariate statistical methods. By this approach, cuticular hydrocarbon profiles of social insects have been shown to be species- (e.g., Kaib et al., 1991; Page et al., 2002), caste- (e.g., Bagnères et al., 1990; Klochkov et al., 2005), and colony-specific (e.g., Butts et al., 1995; Lorenzi et al., 1997).

In the present study, we investigated the role of hydrocarbons as a short-range sex pheromone in *L. distinguendus* by combining chemical analyses and behavioral bioassays. First, we showed that hydrocarbon fractions from females, as well as from freshly emerged males and pupae of either sex, can mediate male courtship behavior. Second, we compared the composition of behaviorally active hexane fractions with inactive ones from older males by principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) (Wold et al., 1989). The latter method was used to characterize chemical components that contribute strongly to the differentiation of active and inactive hydrocarbon profiles. The results are discussed with respect to putative function of these chemicals as constituents of the courtship pheromone of *L. distinguendus*.

Methods and Materials

Insects Parasitoids were reared on larvae of the granary weevil, *Sitophilus granarius*, as described by Steidle and Schöller (1997), at 25°C, 60% RH, and a photoperiod of 16:8

(L/D). To obtain naive parasitoids, single grains containing parasitoids about to emerge were transferred to 1.5-ml microcentrifuge tubes (Sarstedt, Nümbrecht, Germany) and kept under rearing conditions. Emerging parasitoids were held in single-sex groups of 10 individuals, in Petri dishes lined with moistened filter paper, until used in the experiment.

General Procedures for Bioassay The experiment was conducted in a bioassay chamber (10 mm diameter×3 mm height) as described elsewhere (Ruther et al., 2000). Behavioral parameters were observed under a stereo microscope, with illumination by a microscope light, and recorded by using the computer software The Observer 3.0 (Noldus Information Technology, Wageningen, The Netherlands). Males were tested 2–3 d after emergence. One hour before being used in the bioassay, parasitoids were placed individually in micro-centrifuge tubes and kept at ambient temperature. The following behavioral parameters were recorded: (1) Arrestment time, the time males stayed on the sample, (2) antennation time, the time males explored the sample by regular, alternating movements of the antennae, and (3) wing fanning behavior, characteristic high-frequency wing fanning shown by males in the presence of the sex pheromone (Ruther et al., 2000).

Preparation of Hydrocarbon Fractions Batches of 10 individuals each of: (1) freshly emerged females, (2) freshly emerged males, (3) 72-hr-old females, (4) 72-hr-old males, and (5) yellowish pupae from *L. distinguendus* were extracted for 4 d with 60 μl dichloromethane at room temperature. Resulting extracts were concentrated under a gentle stream of nitrogen to 15 μl, applied to a 25-mg silica gel cartridge for solid phase extraction (IST, Mid-Glamorgan, UK) and eluted with 100 μl of hexane. The volume of each hydrocarbon fraction was concentrated under nitrogen to 20 μl (0.5 individual equivalent per microliter) and stored at −80°C until used for bioassay and chemical analysis.

Bioassay: Activity of Hydrocarbon Fractions The behavioral responses of *L. distinguendus* males to filter paper disks (5 mm diameter) treated with four individual equivalents of the five different hexane fraction types (see above) was investigated. After leaving the solvent to evaporate for 15 min, single paper disks were offered to a male in the bioassay chamber, and arrestment time, antennation time, and wing-fanning behavior to the paper disks were recorded over 5 min. For the control, filter paper disks were treated with solvent. Each wasp was tested only once. Males that did not respond to the filter paper disk by wing-fanning behavior were released into another bioassay chamber containing an unmated *L. distinguendus* female. Males that did not show wing fanning in this control test were discarded from the data set. After five parasitoids had been tested, the paper disk was renewed. The bioassay chamber was cleaned regularly with ethanol and deionized water to remove contamination by the walking males. Ten parasitoids were tested to each treatment.

Chemical Analysis Hydrocarbon fractions (N=10 for adults, N=5 for pupae) were analyzed by gas chromatography-mass spectrometry (GC-MS) on a Fisons GC 8060 equipped with a 30 m×0.32 mm DB-5ms fused silica column (film thickness 0.25 μm) and connected to a Fisons MD 800 quadrupole MS (Thermo Finnigan, Egelsbach, Germany). Helium was used as carrier gas, with an inlet pressure of 10 kPa. The temperature program was started at 150°C, raised by 2°C/min to a final temperature of 280°C, and then held for 30 min.

One microliter of each hexane fraction, representing 0.5 of an individual equivalent, was injected together with 25 ng of tetracosane as an internal standard. Relative retention indices (LRI) of methyl-branched and unsaturated hydrocarbons were estimated by coinjection of straight-chain hydrocarbons. Methyl-branched hydrocarbons were identified

by diagnostic ions resulting from the favored fragmentation at the branching points (Lockey, 1988; Nelson, 1993) and by comparing LRI values with literature data (Carlson et al., 1998). Double bond position was determined by iodine-catalyzed methylthiolation using dimethyl disulfide (Francis and Velant, 1981; Howard, 1993). Peak areas for each compound were calculated and related to total peak area for each run.

Statistical Analysis Statistical analysis for the behavioral experiment was performed with the software package Statistica release 4.5 (StatSoft, Tulsa, OK, USA). Arrestment time and antennation time of males on filter paper disks treated with hexane fractions or solvent were analyzed by Mann–Whitney *U* test. Numbers of males responding to the paper disks with wing-fanning behavior were analyzed by a 2×2 Chi^2 test.

The quantitative composition of the five different hydrocarbon fraction types was evaluated by PCA and PLS-DA by using the software program SIMCA-P 10.5 (Umetrics AB, Umeå, Sweden) (Wold et al., 1989; Eriksson et al., 2001). PCA and PLS-DA were conducted as described in detail by Mumm et al. (2004) to extract and display the systematic variation in the data set consisting of 48 different hydrocarbon peaks (Table 1) (Eriksson et al., 2001). In PCA, so-called "scores" are obtained by projecting data observations onto model planes, which are defined by the extracted principal components.

Raw data (integrated peak areas) were normalized, i.e., peak areas of all analyzed compounds (*X* variables) were summed and the relative amount of each variable was calculated. The normalized data were transformed to log (X+0.00001). The constant 0.00001 was added to provide nondetectable components with a small nonzero value (Sjödin et al., 1989). Transformed variables were then mean-centered, Pareto-scaled, and represented as a matrix X. Pareto scaling gives each variable a variance equal to its standard deviation by dividing by the square root of the standard deviation of each column (Eriksson et al., 2001). The ellipse shown in score plots defines the Hotelling's T^2 confidence region (95%). The number of significant principal components was determined by cross-validation (Wold et al., 1989; Eriksson et al., 2001).

In PLS-DA, the data set is modeled in a way similar to PCA, but in combination with a discriminant analysis. The objective of PLS-DA is to find a model that discriminates the *X* data, according to pheromone activity, as well as possible (Eriksson et al., 2001). In contrast to PCA, PLS-DA is a supervised technique, so class memberships of the observations need to be predefined. Therefore, an additional Y matrix was made up with G columns containing the values 1 and 0 as dummy variables for either behaviorally active group or nonactive parasitoid group, respectively. In addition, we calculated the variable importance in the projection (VIP), which is a numerical value describing the importance of the *X* variables, both for the *X* and the *Y* parts (Wold et al., 1993, 2001). Variables with VIP values larger than 1 are most influential for the model (Eriksson et al., 2001; Paolucci et al., 2004).

Results

Bioassay: Activity of Hydrocarbon Fractions Hydrocarbon fractions from freshly emerged males and females, and those from 72-hr-old females and pupae, caused arrestment and stimulated wing fanning in responding males when compared to the solvent control (Fig. 1a–c). In some cases, even more complex elements of male courtship behavior, such as antennal stroking and copulation attempts, were observed during the test period (data not

Table 1 Relative composition of hexane fractions from different life stages of *L. distinguendus*

No.	Compound	LRI[a]	Diagnostic Ions	F0d[c]	M0d[c]	F3d[c]	M3d[c]	YP[c]
01	C25	2500	352	0.08±0,03	0.15±0.07	0.09±0.04	0.14±0.09	0.08±0.04
02	5-MeC25	2550	84/85, 308/309	0.07±0.04	0.27±0.15	0.10±0.04	0.39±0.19	0.17±0.13
03	5,9-DiMeC25	2582	337	0.09±0.03	0.08±0.04	0.03±0.02	0.05±0.03	0.04±0.01
04	C27:1(9)	2672	472, 173, 299 (DMDS)[b]	0.11±0.03	0.11±0.02	0.06±0.03	0.00	0.02±0.02
	+ 3-MeC26		380, 351					
05	4,8-DiMeC26	2691	379 (M-15), 351, 140/141, 280/281	0.00	0.01±0.01	0.00	0.00	0.00
06	C27	2700	380	0.87±0.17	1.02±0.15	0.36±0.09	0.10±0.05	0.15±0.12
07	11-MeC27	2733	168/169, 252/253	0.15±0.04	0.17±0.05	0.07±0.02	0.05±0.04	0.05±0.02
08	5-MeC27	2750	84/85, 336/337	0.06±0.01	0.08±0.01	0.00	0.01±0.01	0.01±0.00
09	3-MeC27	2773	364/365	7.47±1.35	5.04±0.94	1.76±2.16	0.03±0.01	0.98±0.98
10	3,7-DiMeC27	2808	393, 56/57, 379, 126/127, 308/309	1.44±0.31	0.97±0.20	0.29±0.14	0.00±0.00	0.13±0.13
11	squalene	2822	-	0.33±0.08	0.66±0.31	0.55±0.33	0.84±0.50	0.50±0.21
12	2-MeC28	2858	365	0.18±0.03	0.12±0.02	0.18±0.05	0.15±0.02	0.08±0.08
13	C29:1(9)	2873	500, 173, 327 (DMDS)[b]	0.23±0.08	0.21±0.07	0.05±0.02	0.06±0.05	0.05±0.02
14	C29:1(7)	2882	500, 145, 355 (DMDS)[b]	0.02±0.01	0.03±0.01	0.00	0.00	0.00
15	4,8-DiMeC28	2890	407 (M-15), 70/71, 379, 140/141, 308/309	0.06±0.02	0.03±0.01	0.03±0.02	0.00	0.01±0.01
16	C29	2900	408	1.15±0.21	1.25±0.26	0.28±0.09	0.40±0.62	0.33±0.20
17	13-MeC29	2931	196/197, 252/253	0.44±0.09	0.45±0.06	0.08±0.03	0.02±0.02	0.04±0.03
	+ 11-MeC29	2932	168/169, 280/281					
	+ 9-MeC29	2934	140/141, 308/309					
18	7-MeC29	2939	112/113, 336/337	0.05±0.01	0.06±0.01	0.00	0.02±0.03	0.01±0.01
19	6-MeC29	2950	84/85. 364/365	0.14±0.02	0.15±0.02	0.03±0.01	0.09±0.07	0.04±0.04
20	3-MeC29	2972	393	0.72±0.11	0.56±0.09	0.15±0.05	0.00	0.06±0.06
21	5,17-DiMeC29	2976	421 (M-15), 84/85, 379, 196/197, 266/267	0.14±0.03	0.12±0.01	0.03±0.01	0.04±0.01	0.02±0.01
22	C30	3000	422	0.04±0.01	0.13±0.04	0.05±0.06	0.05±0.04	0.05±0.01
23	3,7-DiMeC29	3010	421, 56/57, 406/407, 126/127, 336/337	0.30±0.05	0.25±0.04	0.05±0.02	0.00	0.02±0.02
24	unknown	3033	421 (M-15), 84/84, 351	0.12±0.02	0.15±0.01	0.03±0.02	0.02±0.02	0.02±0.01
25	C31:1(9)	3063	528, 173, 355 (DMDS)[b]	0.56±0.23	0.57±0.16	0.02±0.02	0.06±0.02	0.06±0.06
26	C31	3100	436	0.51±0.07	0.80±0.07	1.31±0.36	1.10±1.53	0.87±0.63
27	15-MeC31	3132	224/225, 252/253	2.64±0.28	2.72±0.93	1.68±0.31	2.58±0.26	1.15±0.98

Table 1 (continued)

No.	Compound	LRI[a]	Diagnostic Ions	F0d[c]	M0d[c]	F3d[c]	M3d[c]	YP[c]
	+ 13-MeC31		196/197, 280/081					
	+ 11-MeC31		168/169, 308/309					
28	3-MeC31	3173	421	0.37±0.05	0.40±0.03	0.21±0.04	0.26±0.03	0.11±0.11
29	5,9-DiMeC31	3182	449(M-15), 84/85, 406/407, 154/155, 336/337	0.16±0.02	0.14±0.01	0.10±0.02	0.23±0.04	0.08±0.09
30	3,7,11-TriMeC31	3234	463 (M-15), 449, 126/127, 378/379, 196/197, 308/309	0.91±0.11	0.78±0.07	0.71±0.12	1.00±0.06	0.39±0.44
31	3,7,11,15-TetraMeC31	3257	492, 463, 126/127, 393, 196/197, 323, 266/267, 252/253	1.21±0.07	1.29±0.08	1.11±0.15	1.57±0.07	0.59±0.70
32	C33:1(9)	3277	556,173, 383 (DMDS)[b]	1.10±0.07	1.12±0.10	0.94±0.12	1.47±0.09	0.54±0.63
33	4,8-DiMeC32	3289	463 (M-15), 435, 140/141, 365	0.62±0.11	0.41±0.03	1.88±0.32	2.01±0.25	0.90±0.96
34	C33	3300	464	0.62±0.07	0.32±0.04	0.89±0.14	0.99±0.10	0.43±0.46
35	15-MeC33	3332	478, 224/225, 280/281	9.93±0.47	14.10±0.59	11.96±2.01	14.89±0.63	6.02±6.87
	+ 13-MeC33		478, 196/197, 308/309					
	+ 11-MeC33		478, 168/169, 337/338					
36	11,21-DiMeC33	3359	492, 168/169, 350/351, 196/197, 322/323	12.56±0.53	18.94±0.58	15.16±2.83	24.17±1.10	8.77±10.48
37	3-MeC33	3376	478, 449	3.10±0.30	2.41±0.15	2.01±0.23	1.46±0.13	0.79±0.88
38	5,9-DiMeC33	3382	492, 84/85, 435, 154/155, 364/365	1.42±0.23	1.53±0.09	2.84±0.20	3.00±0.09	1.24±1.53
39	3,7-DiMeC33	3406	492, 463, 126/127, 392/393	6.49±0.29	4.15±0.28	7.38±0.26	6.76±0.41	3.02±3.71
40	3,7,11-TriMeC33	3431	506, 477, 126/127, 407, 196/197, 336/337	6.25±0.31	4.05±0.31	6.80±0.45	4.18±0.52	2.45±2.92
41	3,7,11,15-TetraMeC33	3460	520, 491, 126/127, 421, 196/197, 351, 266/267, 280/281	18.37±1.14	11.07±0.61	18.68±1.48	10.13±0.99	6.38±7.93
42	C35:1(9)	3481	584, 173, 411 (DMDS)[b]	0.33±0.14	0.11±0.02	1.51±0.23	1.07±0.17	0.60±0.65
43	4,8-DiMeC34	3493	506, 70/71, 463, 140/141, 392/393	0.32±0.10	0.07±0.02	0.68±0.11	0.54±0.08	0.29±0.30

44	17-MeC35	3530	506, 252/253, 280/281	2.29±0.17	3.15±0.19	1.79±0.20	2.30±0.22	0.94±1.03
	+ 15-MeC35		506, 224/225, 308/309					
	+ 13-MeC35		506, 196/197, 336/337					
	+ 11-MeC35		506, 168/169, 364/365					
45	13,17-DiMeC35	3554	520, 196/197, 350/351, 266/267, 280/281	11.70±1.05	15.74±0.68	12.90±1.19	13.25±0.43	5.69±6.75
	+ 11,15-DiMeC35	3556	505 (M-15), 168/169, 378/379, 238/239, 308/309					
46	3-MeC35	3574	506, 477	1.78±0.13	1.17±0.05	2.52±0.19	1.59±0.11	0.89±1.11
	+ 5,9-DiMeC35	3578	520, 463, 393					
47	3,7,11,15-TetraMeC35	3644	548, 519, 126/127, 449, 196/197, 379, 266/267, 308/309	1.43±0.15	1.05±0.11	1.64±0.26	1.14±0.12	0.65±0.70
48	19-MeC37	3722	534, 280/281, 294/295	1.09±0.19	1.84±0.18	1.00±0.21	1.80±0.12	0.66±0.73
	+ 17-MeC37		534, 252/253, 322/323					
	+ 15-MeC37		534, 224/225, 350/351					
	+ 13-MeC37		534, 196/197, 378/379					
	+ 11-MeC37		534, 168/169, 407					

F0d=Fresh females, M0d=Fresh males, F3d=72-hr females, M3d=72-hr males, YP=Yellowish pupae

[a] LRI=Linear retention index

[b] DMDS=diagnostic ions after derivatization with dimethyl disulfide

[c] Means ± SE

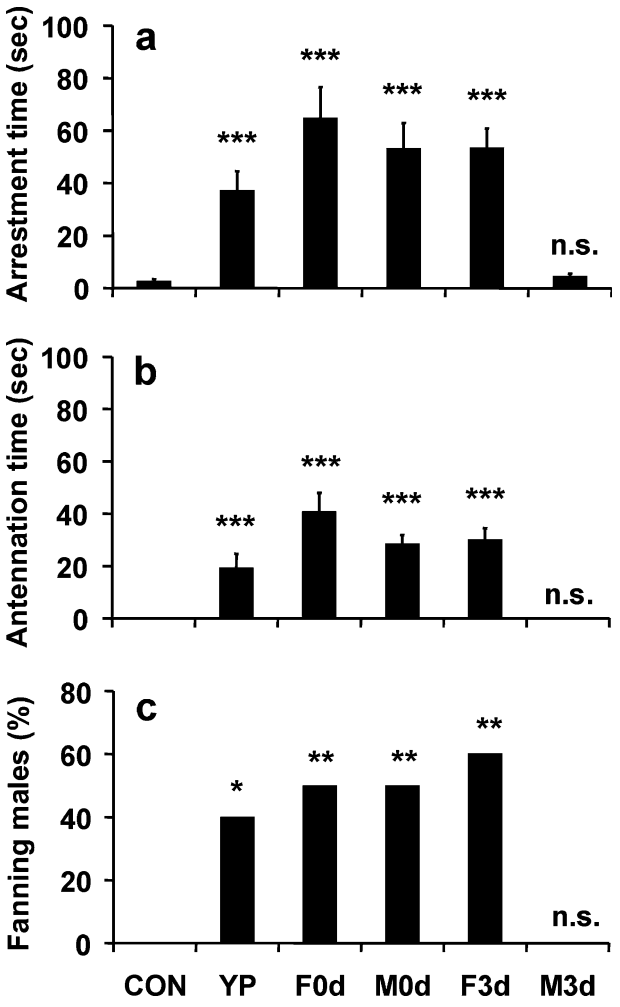


Fig. 1 Response of *L. distinguendus* males to filter paper disks treated with hexane fractions of cuticle extracts from freshly emerged females (F0d) and males (M0d), 72-hr-old females (F3d) and males (M3d), yellowish pupae (YP), and solvent control (CON). (a) Mean arrestment time (±SE), (b) mean antennation time (±SE), and (c) percentages of males showing wing-fanning behavior. Asterisks indicate significant preferences for fractions when compared to control (***$P<0.001$, **$P<0.01$, *$P<0.05$; n.s.=not significant). Mean arrestment times and antennation times were compared by Mann–Whitney *U* tests; wing-fanning behavior was analyzed by 2×2 chi-square tests ($N=10$)

shown). In contrast, males did not respond to hydrocarbon fractions from 72-hr-old males or to control paper disks.

Chemical and Statistical Analysis The 64 compounds identified in the hexane fractions were exclusively cuticular hydrocarbons with chain lengths between 25 and 37 carbon units (Table 1). Hydrocarbons of *L. distinguendus* were comprised of homologous series of *n*-alkanes (C_{25}–C_{33}), monomethyl alkanes (19-, 17-, 15-, 13-, 11-, 7-, 3-methyl), dimethyl alkanes (4,8-, 3,7-, and 5,9-dimethyl), trimethyl alkanes (3,7,11-trimethyl), tetramethyl alkanes (3,7,11,15-tetramethyl), and monoenes with double bonds at position 9 or 7. Additionally, squalene was found as a trace component in all life stages of both sexes. Hydrocarbon profiles were dominated by methyl-branched alkanes with odd carbon chains. The most prominent compounds were 3,7,11,15-TetraMeC_{33}, 11,21-DiMeC_{33}, and 13,17-DiMeC_{35}. Fractions that stimulated male courtship in bioassays showed quantitative but no qualitative differences. In behaviorally inactive fractions of 72-hr-old males, however, a series of hydrocarbons present in the active fractions was missing (Fig. 2).

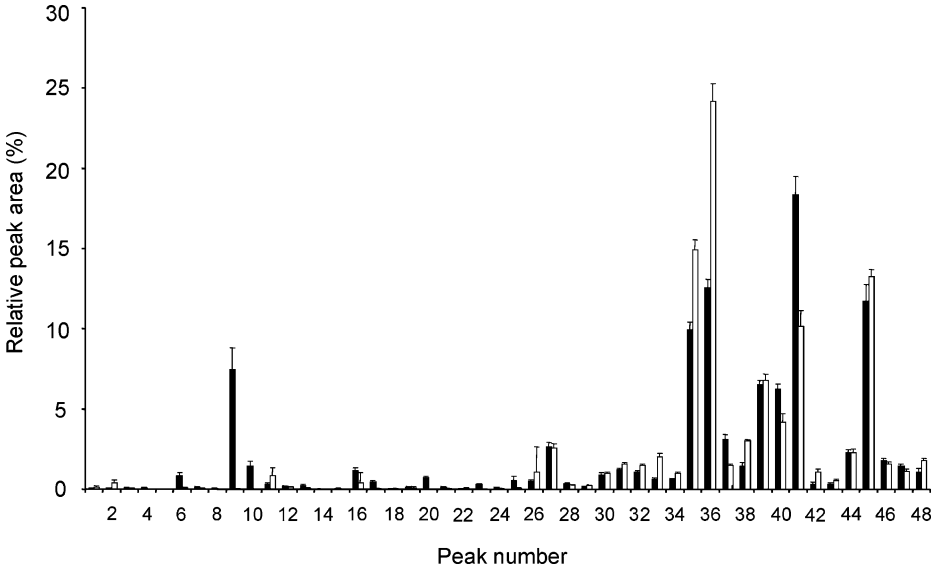


Fig. 2 Relative peak areas of cuticular hydrocarbons from freshly emerged *L. distinguendus* females (black bars; highest bioactivity) and 72-hr-old males (white bars, no bioactivity). Peak numbers correspond to those in Table 1

A PCA, based on relative peak areas found in the five different types of hydrocarbon fractions, was conducted. In this model, four principal components were extracted, explaining a total variation (R^2X) of 79%. A score plot of the first two principle components (PCs) showed that the cuticular hydrocarbon composition was different among all groups (Fig. 3).

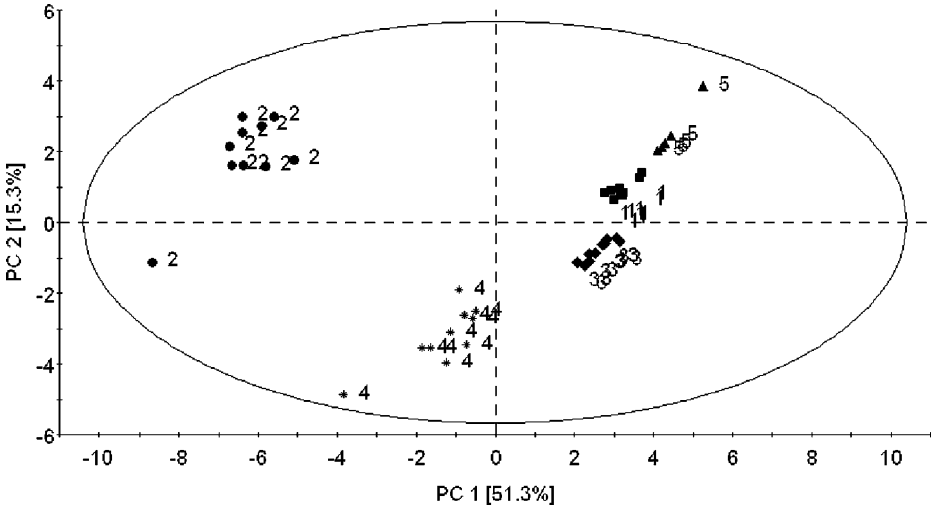


Fig. 3 Analysis of the cuticular hydrocarbon profiles from different *L. distinguendus* life stages. Score plot from principal component analysis (PCA) based on relative amounts of all analyzed hydrocarbons shown in Table 1; 66.5% of the variance in the data is explained by the two first significant principal components, as judged by cross-validation. The ellipse shown in the score plot defines the Hotelling's T^2 confidence interval (95%). 1=freshly emerged males; 2=72-hr-old males; 3=freshly emerged females; 4=72-hr-old females; 5=yellowish pupae. Data were subjected to log-transformation, mean centering, and Pareto scaling

The samples of 72-hr-old male parasitoids were dissimilar to all other samples, as indicated by the projection on the left hand side of the plot. The first PC mainly divided samples of different age, whereas the second PC tended to separate gender of adult parasitoids.

To elucidate which chemical compounds may be responsible for pheromonal activity a PLS-DA was performed. Two parasitoid classes were constructed based on pheromonal activity in the bioassays. Hydrocarbon fractions of freshly emerged males, freshly emerged females, 72-hr-old females, and pupae were combined in one class, and 72-hr-old males represented the second. PLS-DA resulted in a model with three significant discriminant components with R^2X of 70.9%, R^2Y of 99.5%, and Q^2Y of 98.7%. Q^2Y denotes the predictive power of the model, i.e., how class membership can be predicted by the model. The PLS score plot showed that the hydrocarbon fractions of 72-hr-old male parasitoids, which are not behaviorally active, are separated from the active groups (Fig. 4a). The corresponding loading plot depicts which chemical variables contribute strongly to separation of classes. Variables projected close to the dummy variables Y (shown as active and not active) contribute strongly to class separation, and thus have a high discriminatory power (Fig. 4b). A more quantitative way to estimate variable influence is described by the VIP-parameter. Chemical variables most important for resolving behaviorally active parasitoid groups have VIP-parameter values above 1 (Eriksson et al., 2001). Compounds showing VIP-parameter > 1 are, in descending order, peak numbers 10, 20, 23, 4, 14, 9, 17, 15 (data not shown). These compounds are marked with an asterisk in Fig. 4b.

Our PLS-DA model resulted in three significant PLS components. However, with G=2 well-separated classes one expects G - 1 significant PLS components (Eriksson et al., 2004); more components can indicate presence of subclusters. In our model, the higher number of expected components was presumably caused by the samples from the 72-hr-old females. The hydrocarbon composition of these samples was slightly different from the composition of the freshly emerged parasitoids and pupae. This might be ascribed to the age of the parasitoids, which apparently influenced the composition of hydrocarbons (Figs. 3 and 4a).

Discussion

This study reports the characterization of individual cuticular hydrocarbons with respect to their potential role as a short-range sex pheromone in *L. distinguendus*. Behavioral experiments that used hydrocarbon fractions from different life stages demonstrated pheromonal activity from females and from freshly emerged males and immature stages of both sexes. In contrast, there was no pheromonal activity from extracts of 72-hr-old males. Chemical analysis by GC-MS revealed qualitative and quantitative differences in the hydrocarbon profiles of behaviorally active and inactive fractions of *L. distinguendus*. PLS-DA was applied to assess which hydrocarbons are of particular importance in separating the different life stages by pheromone activity. Methyl-branched alkanes (3-MeC27, 3,7-DiMeC27, 4,8-DiMeC28, 3-MeC29, 3,7-DiMeC29, and 13-MeC29) had the strongest impact in the discriminant analysis. However, some alkenes (C27:1(9), C29:1 (7)) also had a high discriminating power. Apart from 3-MeC27, all chemicals that have been found to possess high discriminating power are minor components of the bioactive hydrocarbon profiles. Some of these compounds were totally absent in the profiles of (inactive) extracts from 72-hr-old males (Table 1, Fig. 2) and thus are good candidates for

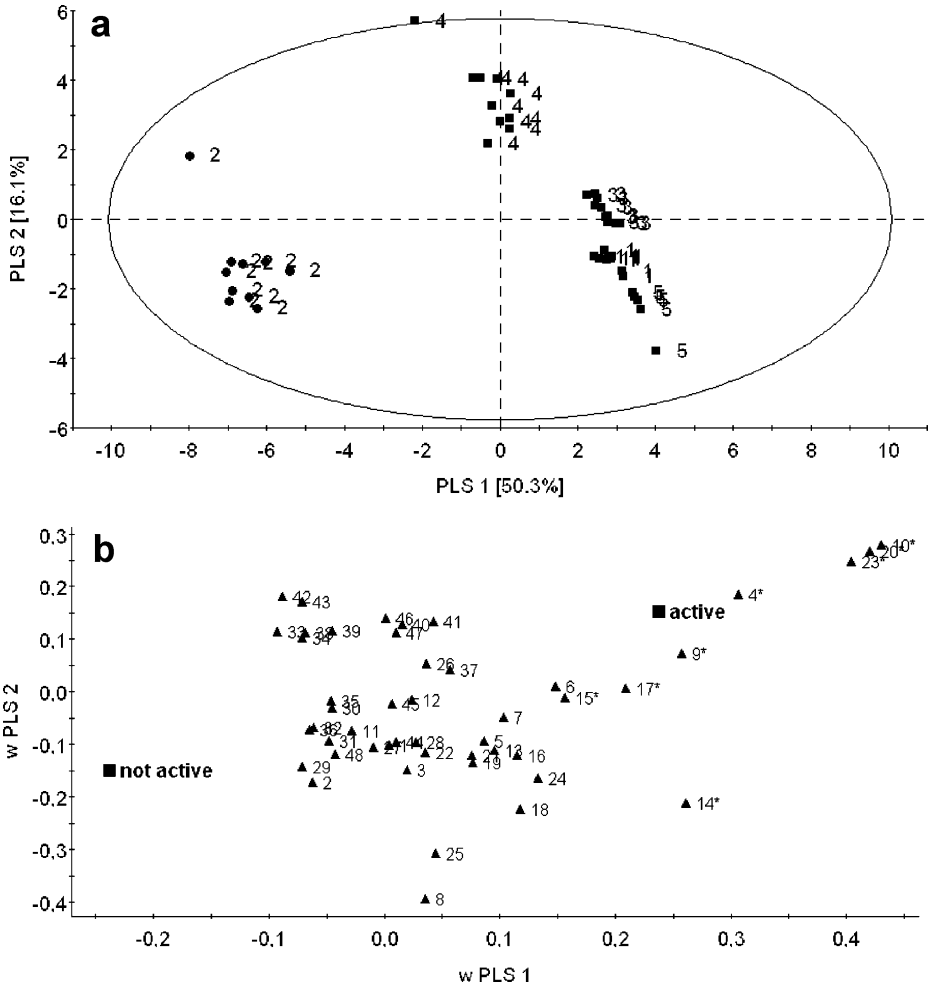


Fig. 4 Analysis of the cuticular hydrocarbon profiles from different *L. distinguendus* life stages. (a) Score plot and (b) loading plot from partial least squares-discriminant analysis (PLS-DA) based on relative amounts of all analyzed hydrocarbons shown in Table 1. A total of 66.4% of the variance of the *X* variables and 98.5% of the variance of the dummy variable *Y* is explained by the two significant principal components, as judged by cross-validation. *Y* variables are shown as "active" or "not active". 1=freshly emerged males; 2=72-hr-old males; 3=freshly emerged females; 4=72-hr-old females; 5=yellowish pupae; box=no pheromone activity; dot=pheromone activity. Data were subjected to log-transformation, mean centering, and Pareto scaling

mediating pheromone activity in *L. distinguendus*. In recent years, the number of studies on the role of cuticular hydrocarbons as insect semiochemicals has grown immensely (reviewed by Howard and Blomquist, 2005). Evidence is increasing that cuticular hydrocarbons play a general role as courtship pheromones in parasitoids, by mediating arrestment and other, more complex behavioral elements. However, few studies have identified which cuticular hydrocarbons are bioactive. Syvertsen et al. (1995) reported a series of female-specific C_{25}–C_{35} (*Z*,*Z*)-alkadienes with pheromonal properties in the braconid wasp *Cardiochiles nigriceps*. In the ichneumonid parasitoid *Eriborus terebrans*,

Shu and Jones (1993) reported a synergism between a polar female-derived sex pheromone and nonpolar components, most probably cuticular hydrocarbons. Apart from *L. distinguendus*, evidence for the function of cuticular hydrocarbons as courtship pheromones has been provided in two other pteromalids. Sullivan (2002) identified 28 compounds, consisting of aliphatic and methyl-branched alkanes with up to two methyl groups, in female extracts of *Roptrocerus xylophagorum* that correlated with the male response. Steiner et al. (2006) found numerous gender-related differences in *N. vitripennis* when comparing the composition of bioactive hydrocarbon fractions from females with inactive ones from males.

Our bioassays with hydrocarbon fractions from different life stages support recent investigations showing that *L. distinguendus* produces sex pheromone during pupal development (Steiner et al., 2005). The authors demonstrated that searching *L. distinguendus* males were arrested on parasitized grains containing females about to emerge. A similar phenomenon has been reported in *Anisopteromalus calandrae* (Pteromalidae) (Yoshida, 1978) and *Apanteles glomeratus* (Braconidae) (Tagawa, 1977). This pre-emergence pheromone release may increase the chances of females being inseminated before leaving the emergence site to search for hosts.

Interestingly, bioactive hydrocarbons are also present in developing and newly emerged males of *L. distinguendus*. After emergence, males become less bioactive within 32 hr, suggesting the production and/or degradation of the bioactive hydrocarbons is regulated differently from that in female parasitoids, which remain bioactive to courting males. The mechanism of this deactivation is not yet understood. However, we demonstrated that only living males lose bioactivity; dead males stored under the same conditions had bioactivity for several days. This suggests that the loss of pheromonal activity in males is caused by an active process rather than simple evaporation of the bioactive compounds. This may be achieved by selective metabolism or translocation of individual hydrocarbons from the cuticle to inner regions of the insect. In termites, for example, radiolabeling experiments have demonstrated that topically applied hydrocarbons are transported to the hemolymph by the lipid carrier protein lipophorin (Sevala et al., 2000).

Developing *L. distinguendus* males may benefit from possessing the pheromone. Steiner et al. (2005) showed that searching males are unable to distinguish between grains containing female or male conspecifics that are about to emerge. They suggested that developing males inside the grains might fool their already emerged competitors by distracting them away from searching for actual females. Another parasitoid species in which young males elicit courtship behavior in male conspecifics is the ichneumonid *Itoplectis conquisitor*. Robacker et al. (1976) demonstrated that extracts of both freshly emerged males and females elicit sexual behavior in older males. However, bioactive constituents of this multicomponent pheromone are terpene aldehydes rather than hydrocarbons (Robacker and Hendry, 1977).

By applying discriminant analysis to the hydrocarbon composition of parasitic Hymenoptera, we identified a set of compounds that allow discrimation of bioactive from bio-inactive life stages and which may be responsible for pheromonal activity in *L. distinguendus*. As our findings are based on mathematical modeling, future studies will focus on behavioral experiments using synthetic reference compounds to ascertain whether these hydrocarbons function as courtship pheromone in *L. distinguendus*.

Acknowledgments This research was funded by the Deutsche Forschungsgemeinschaft (Ru 717/5-1). R.M. was funded by the European Commission contract MC-RTN-CT-2003-504720 “ISONET”.

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J Chem Ecol (2007) 33:839–847
DOI 10.1007/s10886-007-9259-4

(11*Z*,13*E*)-Hexadecadien-1-yl Acetate: Sex Pheromone of the Grass Webworm *Herpetogramma licarsisalis*—Identification, Synthesis, and Field Bioassays

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Received: 15 August 2006 / Revised: 3 January 2007 / Accepted: 13 January 2007 /
Published online: 2 March 2007
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Abstract The grass webworm *Herpetogramma licarsisalis* (Lepidoptera: Crambidae), which has recently established in pasture in Northland, New Zealand, is an important pest of many tropical and subtropical grasses. Two pheromone components, (*Z*)-11-hexadecen-1-yl acetate (*Z*11–16:Ac) and (11*Z*,13*E*)-hexadecadien-1-yl acetate (*Z*11,*E*13–16:Ac), were identified in pheromone gland extracts of female moths by gas chromatography (GC), GC-electroantennographic detection, and GC-mass spectrometry in conjunction with micro-chemical tests (dimethyldisulfide and 4-methyl-1,2,4-triazoline-3,5-dione derivatizations). *Z*11,*E*13–16:Ac and its geometric isomer (11*E*,13*Z*)-hexadecadien-1-yl acetate (*E*11,*Z*13–16:Ac) were synthesized via stereoselective Wittig reactions, and the identity of the diene present in the pheromone glands was confirmed to be *Z*11,*E*13–16:Ac. Field bioassays at Indooroopilly in Brisbane, Australia, established that *Z*11,*E*13–16:Ac was necessary and sufficient for attraction of male grass webworm moths and that the corresponding alcohol, (11*Z*,13*E*)-hexadecadien-1-ol (*Z*11,*E*13–16:OH), had a strong inhibitory effect on trap catches at the ratios tested. When mixed with *Z*11,*E*13–16:Ac in various ratios, *Z*11–16:Ac had no effect on the attractiveness of lures.

Electronic supplementary material The online version of this article (doi:10.1007/s10886-007-9259-4) contains supplementary material, which is available to authorized users.

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Keywords Grass webworm · *Herpetogramma licarsisalis* · Crambidae · (*Z*)-11-Hexadecen-1-yl acetate · (11*Z*,13*E*)-Hexadecadien-1-yl acetate · (11*Z*,13*E*)-Hexadecadien-1-ol · Behavioral antagonist · MTAD

Introduction

The grass webworm *Herpetogramma licarsisalis* (Walker, 1859) (Lepidoptera: Crambidae) is an important pest of many tropical and subtropical grasses used for pasture and ornamental turf. *H. licarsisalis* is widely distributed globally as it is present in central Africa, Sierra Leone, Egypt, Saudi Arabia, India, Japan, Southeast Asia, Hawaii, and northern Australia (Tashiro, 1976; Common, 1990; Goater and Knill-Jones, 1999). Infestations have also been reported from Spain and Portugal, and the species has recently been recorded in Britain and Sweden (Goater and Knill-Jones, 1999; Svensson, 2003).

In 1999, *H. licarsisalis* larvae were confirmed in kikuyu (*Pennisetum clandestinum* Hochst. Ex Chiov. South) dairy pasture on the Aupouri Peninsula in Northland, New Zealand (Hardwick et al., 2000). During the outbreak, densities of up to 1,500 caterpillars per square meter were recorded that were associated with significant damage to kikuyu swards (S. Hardwick, personal communication). Despite the fact that the infestation was considered to be at the margins of the moth's climatic and geographical range (Hardwick et al., 2000; Willoughby and Barns, 2002; Jensen and Cameron, 2004), low-density grass webworm populations still persist on the Aupouri Peninsula.

Our objective was to identify the sex pheromone of *H. licarsisalis* to use as a monitoring tool of the existing populations for deployment in delimitation surveys and for collection of phenological data for pest management. A pheromone attractant would also be useful as a basis of an early warning system for any future outbreaks in the Northland area, including the detection of new incursions from the east coast of Australia (Fox, 1978).

To date, sex pheromone attractants for more than 40 crambid moth species have been identified (El-Sayed, 2006), but none is known from the genus *Herpetogramma*. We report in this study the identification of two compounds in pheromone gland extracts of *H. licarsisalis* and the evaluation of their biological activity in a series of field bioassays in Queensland, Australia.

Methods and Materials

Insects Approximately 50 *H. licarsisalis* pupae were obtained from AgResearch, Ruakura (see Jensen and Cameron, 2004 for a review). A colony was established at HortResearch, Lincoln, and maintained for five generations at 22°C and 60% relative humidity. Freshly emerged adults were placed in 400×600 mm polyethylene bags (one to two males and one to two females per bag). Each of the bags was supported by a stainless steel wire frame. Each bag contained six 'Pioneer' hybrid maize (*Zea mays* ssp. *mays*) seedlings on which female moths readily deposited egg batches. Maize leaves infested with egg batches were collected daily and placed into 680 ml plastic containers (Huhtamaki Henderson Ltd., Auckland, NZ).

After eclosion, groups of ca. 10 early-instar larvae were transferred to additional plastic containers to avoid cannibalism. The larvae were supplied with fresh maize leaves as required. Pupae were collected and held individually until adult emergence in 35 ml plastic containers (Lily® portion cups with lids; Huhtamaki Henderson Ltd.). The antennae of males were used for coupled gas chromatographic-electroantennographic detection (GC-EAD),

whereas females had their pheromone glands excised and extracted for analysis by GC-EAD, GC, and GC-mass spectrometry (MS).

Pheromone Gland Extraction and Analyses Pheromone glands were removed from actively calling 24- to 48-hr-old female *H. licarsisalis* during the third and fourth hours of the scotophase. Excised glands were extracted in 20–40 μl of *n*-hexane for 5 min. The resulting supernatant was stored at −20°C until analysis. For quantitative analysis, the pheromone glands of 10 48-hr-old females were extracted with a 40-μl aliquot of *n*-hexane containing 4 ng of tetracosane as an internal standard. Two pheromone gland extracts were also derivatized, with either 4-methyl-1,2,4-triazoline-3,5-dione (MTAD; Sigma-Aldrich, NSW, Australia) or dimethyldisulfide (DMDS; Merck, Darmstadt, Germany), to determine the double-bond positions of the two MS-detectable compounds that elicited EAD responses. For the MTAD derivatization of a 26-female gland extract, we followed the procedure of McElfresh and Millar (1999). For the DMDS derivatizations of a 96-female gland extract, and (11*Z*)-hexadecen-1-yl acetate (*Z*11–16:Ac) and (11*E*)-hexadecen-1-yl acetate (*E*11–16:Ac) standards, we followed the procedure described by Buser et al. (1983).

Gas Chromatography-Electroantennographic Detection We analyzed pheromone gland extracts (five batches of 10, 13, 27, 18, and 10 pheromone glands) by GC-EAD, with a Varian 3800 GC coupled to an EAD Recording Unit (Syntech, Hilversum, The Netherlands). For details of the preparation of moth antennae, and our setup of associated equipment for GC-EAD analyses, see the work of Gibb et al. (2006). Extracts were run on DB-5 (30 m × 0.25 mm i.d. × 0.5 μm film; Agilent Technologies, Palo Alto, CA, USA) and DB-Wax columns (30 m × 0.25 mm i.d. × 0.5 μm film; Agilent) with 1:1 split outlets. Helium was used as the carrier gas (1 ml min^{-1}), and injections were splitless for 0.6 min. Injector and detector temperatures were 220° and 300°C, respectively, and the GC temperature program was 80°C for 1 min, 10°C min^{-1} to 240°C, and held for 35 min. Kováts retention indexes (KIs) (Kováts, 1965; Marques et al., 2000) were calculated for the compounds that elicited antennal responses to compare relative retention times and, thus, to obtain rudimentary information about the identity of the EAD-active compounds.

Gas Chromatography-Mass Spectrometry We compared the retention times and mass spectra of the EAD-active compounds detected in a gland extract (1 μl, ca. 18-female equivalents) with those of known standards by using a Varian 3800 GC coupled to a Varian 2200 mass spectrometer (GC-MS). Helium was used as the carrier gas (1 ml min^{-1}), and injections were splitless for 0.6 min. Transfer line and ion trap temperatures were 250° and 200°C, respectively. The GC injector temperature was 220°C, and the temperature program was 100°C for 1 min, 10°C min^{-1} to 210°C, and held for 18 min using a VF-5ms column (30 m × 0.25 mm i.d. × 0.25 μm film; Varian Inc., Walnut Creek, CA, USA). The MTAD- and DMDS-derivatized pheromone gland extracts, and the DMDS-derivatized standards, were also analyzed by GC-MS (injector temperature 250°C; 8 min solvent delay for MS) on the VF-5ms column, using oven ramps of 15°C min^{-1} from 100° to 300°C, and held for 20 min (MTAD), or 15°C min^{-1} from 100° to 260°C, and held for 20 min (DMDS).

Chemicals *Z*11–16:Ac and E11–16:Ac (both >98% isomerically pure by GC analysis) were obtained from Pherobank (Wageningen, The Netherlands). (10*E*,12*Z*)-Hexadecadien-1-yl acetate (*E*10,*Z*12–16:Ac) and (10*E*,12*Z*)-hexadecadien-1-ol (*E*10,*Z*12–16:OH) were available from previous work (Suckling et al., 2005). (11*Z*,13*E*)-Hexadecadien-1-ol (*Z*11,*E*13–16:OH) and the corresponding acetate (11*Z*,13*E*)-hexadecadien-1-yl acetate (*Z*11,*E*13–16:

Ac) were synthesized as shown in Scheme 1 and as described in detail in the online supplement. The synthesis of (11*E*,13*Z*)-hexadecadien-1-yl acetate (*E*11,*Z*13–16:Ac) is also described in the online supplement.

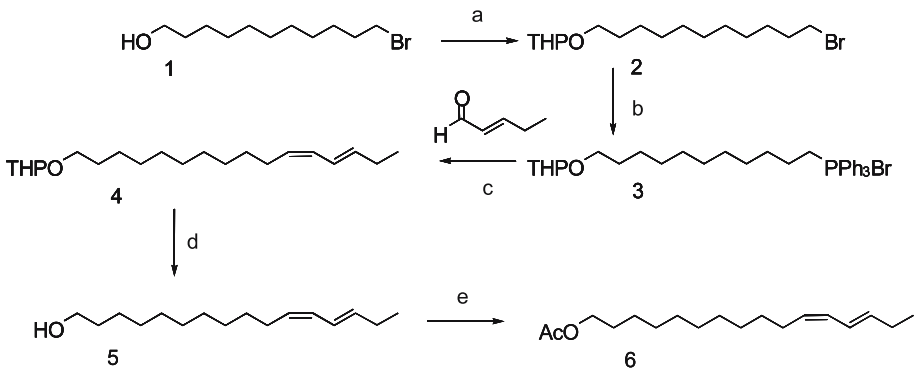
Field Bioassays We undertook a series of field bioassays in kikuyu turf at the Indooroopilly Golf Course (27.5090° S, 152.9948° E), Brisbane, Australia (trials A, B, and C). Test compounds, based on ratios established from GC analyses of *H. licarsisalis* gland extracts, were loaded onto red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA). In all trials, green delta traps (Clare et al., 2000) were attached to fencing wires, at a height of ca. 0.5 m, with 10 m spacings between treatments and >50 m spacings between replicates. Trap positions within each replicate were rerandomized, and sticky inserts were replaced, at each check for capturing male *H. licarsisalis*.

In trial A (April 8–28, 2005), the attraction of *H. licarsisalis* males to various combinations of the three sex pheromone candidate compounds was investigated. Traps were checked seven times at intervals of 1–4 d. Treatments were (1) blank control, (2) 100:0:0, (3) 0:100:0, (4) 0:0:100, (5) 30:70:0, (6) 30:70:1, and (7) 30:70:10 μg loadings of *Z*11–16:Ac, *Z*11,*E*13–16:Ac, and *Z*11,*E*13–16:OH ($N = 5$).

In trial B (January 24 to February 15, 2006), the attraction of *H. licarsisalis* males to 100 μg of *Z*11,*E*13–16:Ac was compared with the attraction of *H. licarsisalis* males to binary mixes comprising 100 μg of *Z*11,*E*13–16:Ac and various doses of *Z*11–16:Ac. Traps were checked six times at intervals of 1–5 d. Treatments were (1) blank control, (2) 100:0, (3) 100:11, (4) 100:25, (5) 100:42, and (6) 100:66 μg loadings of *Z*11,*E*13–16:Ac and *Z*11–16:Ac ($N = 4$).

Trial C was a dose response trial based on treatment 3 from trial A. Traps were checked four times at intervals of 3 d from February 21 to March 5, 2006. Treatments were (1) blank control, (2) 10, (3) 100, and (4) 1000 μg doses of *Z*11,*E*13-16:Ac ($N = 5$).

Statistical Analyses Only treatments that attracted moths were included in the statistical analyses. For each trial, the sum of moths captured per trap was analyzed for treatment and block effects using two-way analysis of variance (ANOVA) (MINITAB, version 14). Residual plots were used to check the validity of the ANOVA assumptions, and square root



Scheme 1 Syntheses of (11*Z*,13*E*)-hexadecadien-1-ol (**5**) and (11*Z*,13*E*)-hexadecadien-1-yl acetate (**6**). Reaction conditions: (**a**) Amberlyst 15, *t*-butylated hydroxyanisole (BHA), 3,4-dihydropyran, diethyl ether, 0°C to room temperature (RT); (**b**) PPh_3, MeCN, reflux; (**c**) sodium ethoxide, THF, reflux then −100°C, addition of (*E*)-2-pentenal, then to RT; (**d**) Amberlyst 15, BHA, MeOH; 45°C; (**e**) Ac_2O/pyridine, RT

($x + 0.5$) transformations of the summed captures were undertaken to remove variance heterogeneity. Means were compared by Fisher's protected least-significant difference test ($P = 0.05$) (MINITAB, version 14).

Results and Discussion

Identification of Gland Components Eliciting Antennal Responses In GC-EAD assays, male *H. licarsisalis* antennae responded repeatedly to three compounds in pheromone gland extracts. Only two of these compounds (X and Y, Fig. 1) were visible in the flame ionization detector (FID) trace (KIs of 2001 and 2037, respectively, on the DB-5 column, and 2365 and 2508, respectively, on the DB-Wax column). Antennal response times were the only indication of the presence of compound Z in the extracts. On the DB-5 column, no peak for compound Z was visible in the FID trace, whereas on the DB-Wax column, the antennal response time suggested that the compound was co-eluting on the tail of another unidentified compound (Fig. 1).

The retention times of compound X on DB-5 and DB-Wax columns suggested the presence of a monounsaturated hexadecen-1-yl acetate (Marques et al., 2000), and KIs were identical with those of the *Z*11–16:Ac standard. In addition, the mass spectrum of compound X [*m*/*z* (relative intensity—rel. int.) 222 (4), 166 (2), 137 (5), 123 (13), 109 (23), 95 (56), 81 (100), 67 (98), 61 (7), and 55 (49)] was virtually identical with that of *Z*11–16:Ac, and the retention time of X was the same as the retention time of *Z*11–16:Ac on the VF-5ms column. A comparison of the retention times of the DMDS adducts of *Z*11–16:Ac and *E*11–16:Ac, with the DMDS derivatization products of a grass webworm pheromone gland extract, unequivocally confirmed that compound X was *Z*11–16:Ac rather than *E*11–16:Ac. Both hexadecen-1-yl acetate DMDS adducts have a molecular ion at *m*/*z* (rel. int.) 376 (7) and diagnostic ions at *m*/*z* (rel. int.) 117 (20, $[C_5H_{10}SCH_3]^+$) and 259 (100, $[O_2C_{13}H_{24}SCH_3]^+$) corresponding to the addition of DMDS to a double bond at position 11.

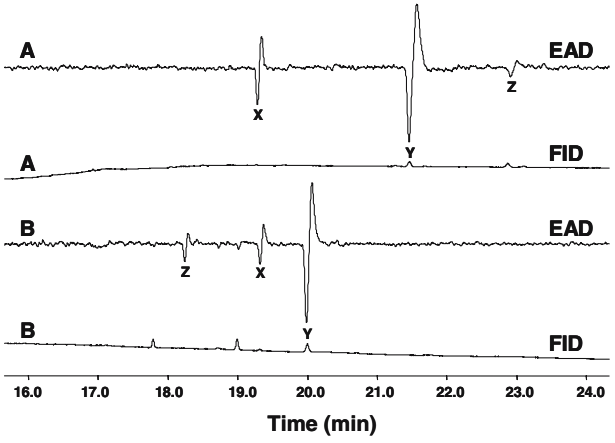


Fig. 1 Coupled gas chromatogram-electroantennograms of *Herpetogramma licarsisalis* male antennae responding to compounds (X = (*Z*)-11-hexadecen-1-yl acetate, Y = (11*Z*,13*E*)-hexadecadien-1-yl acetate, and Z = tentatively identified as (11*Z*,13*E*)-hexadecadien-1-ol), in pheromone gland extracts (ca. 1-female equivalents), eluting from DB-Wax (**A**) and DB-5 (**B**) columns

The retention times of compound Y on the DB-5 and DB-Wax columns suggested a C_{16}-conjugated dien-1-yl acetate after a comparison with a number of C_{16}-conjugated and C_{16}-nonconjugated dien-1-yl acetate standards. Similarly, the antennal response time for compound Z on the DB-5 and DB-Wax columns suggested a C_{16}-conjugated dien-1-ol by comparison with a number of C_{16}-conjugated and C_{16}-nonconjugated dien-1-ol standards. Furthermore, the retention times of *E*10,*Z*12–16:Ac and *E*10,*Z*12–16:OH were the same as those of compounds Y and Z, respectively. Both of these standards elicited strong EAD signals from male grass webworm antennae, and when *E*10,*Z*12–16:Ac was added to an aliquot of pheromone extract, it co-eluted with compound Y on both columns. However, a trapping trial in Northland in early 2003, using these two conjugated dienes in various combinations with *Z*11–16:Ac, failed to trap any moths at a time when moths were observed flying (Willoughby, unpublished data).

We hypothesized that although compounds Y and Z were C_{16}-conjugated dienes, the diene was probably at either the 9,11 or 11,13 position. The analysis of the MTAD-derivatized pheromone gland extract by GC-MS revealed an adduct with a molecular ion at *m*/*z* (rel. int.) 393 (1), and diagnostic ions at *m*/*z* (rel. int.) 364 (21, $[C_{19}H_{30}N_3O_4]^+$) and 194 (100, $[C_9H_{12}N_3O_2]^+$), indicative of a C_{16} dien-1-yl acetate with the diene in the 11,13 position. Thus, compound Y was identified as an 11,13-hexadecadien-1-yl acetate. We could not confirm the identity of compound Z due to the small amount of material in gland extracts (not detectable by GC-FID or GC-MS), but we hypothesized that compound Z was likely to be the C_{16} dien-1-ol precursor of the insect-produced C_{16} dien-1-yl acetate (see Jurenka, 2004 for a review).

Stereoselective syntheses of the (*Z*,*E*) and (*E*,*Z*) isomers of 11,13-hexadecadien-1-yl acetate were carried out. These syntheses also made the (*Z*,*Z*) and (*E*,*E*) isomers as trace by-products, giving us access to all four stereoisomers. A comparison of the spectrum [*m*/*z* (rel. int.) 280 (1, M^+), 220 (4), 177 (3), 163 (4), 149 (8), 135 (15), 121 (27), 107 (14), 95 (43), 81 (54), 67 (100), 55 (21), and 43 (44)] and retention times of compound Y in pheromone gland extract, with the four spectra of the 11,13-stereoisomers, unambiguously confirmed compound Y as *Z*11,*E*13–16:Ac. Analysis of an *H. licarsisalis* gland extract by GC on the DB-Wax column showed that *Z*11,*E*13–16:Ac and *Z*11–16:Ac were present in a ca. 70:30 ratio. The amount of *Z*11,*E*13–16:Ac and *Z*11–16:Ac per female was calculated to be ~280 and 100 pg, respectively ($N = 10$).

Synthesis of Pheromone Compounds The synthesis of (11*Z*,13*E*)-hexadecadien-1-ol **5** (Scheme 1) and the corresponding acetate **6** (Scheme 1) started with 11-bromoundecanol **1**, which was protected using dihydropyran with Amberlyst 15 as catalyst (Santangelo et al., 2002). The THP-protected bromide **2** was transformed to the phosphonium salt **3** with PPh_3 (Legrand et al., 2004). A Wittig reaction between **3** and (*E*)-2-pentenal in the presence of sodium ethoxide made, after deprotection, the (*Z*,*E*)-dien-1-ol **5** as the major product. Acetylation of **5** using acetic anhydride in pyridine produced acetate **6** (Santangelo et al., 2002).

Field Bioassays In the field bioassays, we established *Z*11,*E*13–16:Ac as the main sex pheromone component of *H. licarsisalis*. In trial A, the treatments with *Z*11,*E*13–16:Ac as a single component or in combination with *Z*11–16:Ac (treatments 3 and 5, respectively) were equally attractive to male moths ($P < 0.05$) (Fig. 2a). Single-component lures loaded with *Z*11,*E*13–16:OH were not attractive, and the addition of *Z*11,*E*13-16:OH to the binary mix of *Z*11,*E*13-16:Ac and *Z*11–16:Ac was strongly inhibitory at the ratios tested ($P < 0.05$).

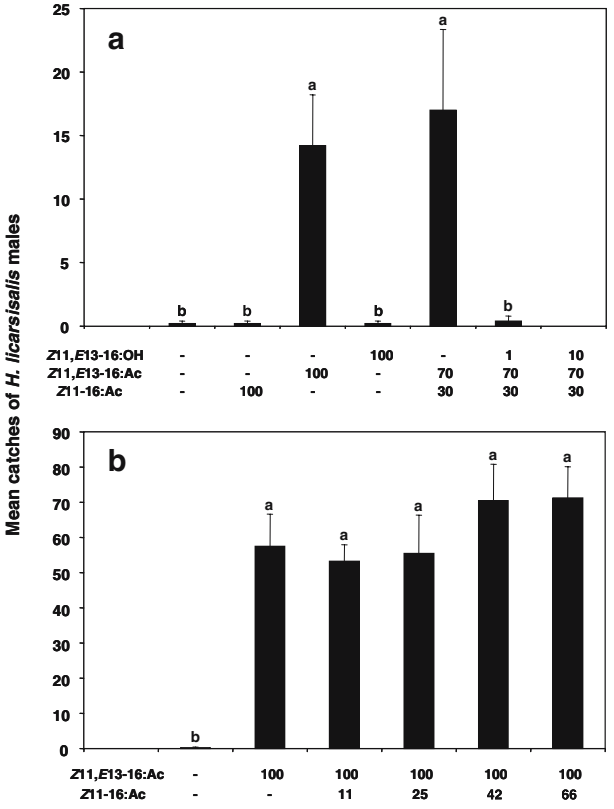


Fig. 2 Mean (+SEM) total catches of *Herpetogramma licarsisalis* male moths at Indooroopilly Golf Course (Brisbane, Australia) in a blend trial (trial A) of the three compounds that elicited responses from moth antennae (two-way ANOVA for treatment effect, $F = 23.18$; $df = 5, 29$; $P < 0.001$, and for block effect $F = 2.9$; $df = 4, 29$; $P = 0.048$, followed by Fisher's protected least significant differences (PLSD test), and in a two-component blend trial (trial B), using (11*Z*,13*E*)-hexadecadien-1-yl acetate and (*Z*)-11-hexadecen-1-yl acetate (two-way ANOVA for treatment effect, $F = 53.29$; $df = 5, 23$; $P < 0.001$, and for block effect $F = 5.4$; $df = 3, 23$; $P = 0.01$, followed by Fisher's PLSD test). Amounts on the *x*-axis are micrograms per lure, and treatments labeled with the *same letter* are not significantly different ($P > 0.05$)

Although we identified *Z*11–16:Ac from gland extracts, male grass webworm moths were not attracted to lures loaded with this compound only. Furthermore, *Z*11–16:Ac, when added to *Z*11,*E*13–16:Ac over a range of ratios, did not increase attraction to lures ($P > 0.05$) (Fig. 2b).

In trial C (Fig. 3), we tested the optimal dose of *Z*11,*E*13–16:Ac required for attracting male grass webworm moths. Lures loaded with 10 μg of *Z*11,*E*13–16:Ac attracted few moths ($P < 0.05$), and a 1,000-μg dose was not better than the 100-μg dose ($P > 0.05$).

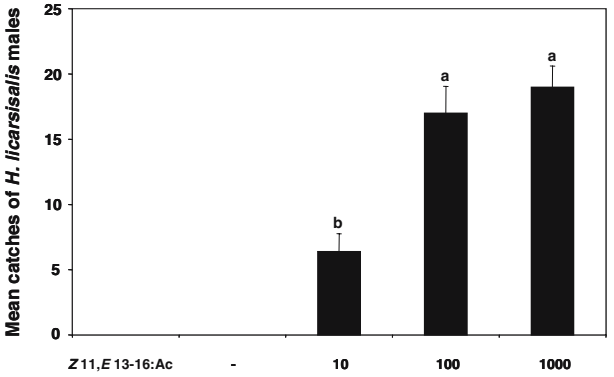


Fig. 3 Mean (+SEM) total catches of *Herpetogramma licarsisalis* male moths to various doses of (11*Z*,13*E*)-hexadecadien-1-yl acetate (trial C) at Indooroopilly Golf Course (Brisbane, Australia) (two-way ANOVA for treatment effect, $F = 25.98$; $df = 2, 14$; $P < 0.001$, and for block effect $F = 2.28$; $df = 4, 14$; $P = 0.149$, followed by Fisher's PLSD test). Amounts on the *x*-axis are micrograms per lure, and treatments labeled with the *same letter* are not significantly different ($P > 0.05$)

*Z*11,*E*13–16:Ac has already been used for monitoring male grass webworm moths in the infested area in Northland. Lures containing 100 μg of *Z*11,*E*13–16:Ac have also successfully attracted male grass webworm moths in Hawaii (Suckling, unpublished data), where the insect is the major pest of turf grass (Tashiro, 1977). Although the 11,13 position for a C_{16}-conjugated diene has previously been reported for pheromone components of moths in the family of Crambidae (Arai et al., 1982; Millar et al., 2002), this is the first identification of an (11,13)-hexadecadien-1-yl acetate as a sex pheromone component of a crambid moth. *Z*11,*E*13–16:Ac also has been found as a minor component in the pheromone glands of two Notodontidae (Bestmann et al., 1991, 1993).

Acknowledgments We thank Julie Hackett, Peter Jones, and Kirsten Moss for the technical assistance in rearing insects, and Barry Bunn and Dave Rogers for commenting on an earlier version of the manuscript. Eric Jang (USDA-ARS) and his team assisted in trapping in Hawaii. Funding was provided by the New Zealand Foundation for Research, Science, and Technology (FRST), under the "Better Border Biosecurity" and previous programs, to develop scientific capability to respond to biosecurity incursions. We are also grateful to the University of Kalmar, Sweden, for the additional financial support.

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J Chem Ecol (2007) 33:849–859
DOI 10.1007/s10886-007-9263-8

Food Wrapping with the Postpharyngeal Gland Secretion by Females of the European beewolf *Philanthus triangulum*

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Received: 7 September 2006 / Revised: 22 January 2007 / Accepted: 26 January 2007 /
Published online: 3 March 2007
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Abstract Ground-nesting animals share their habitat with countless microorganisms that can play important roles as pathogens or competitors for food resources. Thus, species that store food in the soil, either for themselves or for their progeny, must protect these resources against microbial degradation. Females of the European beewolf, *Philanthus triangulum*, hunt honeybees as provisions for their brood and store the paralyzed prey in their subterranean nests. A previous study had shown that females lick the surface of prey before oviposition and that this licking treatment delays mold growth. Here, we showed that females apply large amounts of a secretion from their postpharyngeal glands onto the surface of their prey during the licking behavior. Inhibition-zone assays showed that comparatively large amounts of the gland secretion had no direct antimycotic effect. We discuss our findings with regard to other possible mechanisms of the postpharyngeal gland secretion against fungal growth.

Keywords *Philanthus triangulum* · Hymenoptera · Crabronidae · Honeybee · *Apis mellifera* · Postpharyngeal gland · Prey preservation · (*Z*)-9-Pentacosene

Introduction

Brood-caring insects provision their progeny with highly nutritive food (pollen, paralyzed prey, or carcasses) that must remain consumable for the feeding period of the larvae

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(Vander Wall, 1990; O'Neill, 2001). In particular, ground-nesting species, like many aculeate Hymenoptera, are adversely affected by fungal contamination of their provisions (Eickwort et al., 1981; Cane et al., 1983; Strohm and Linsenmair, 2001). Fungal infestations of the stored resources can decrease the parent's fitness returns, either by killing the larvae (Janzen, 1977) or by decreasing progeny size and reproductive success (Strohm, 2000). Insects should, therefore, have evolved defense mechanisms against fungal infestations of the provisions and their brood. Mostly, antifungal mechanisms are mediated by chemical compounds that interfere with the fungi at different stages of their growth (Gross et al., 1998, 2002; Bot et al., 2002). In the present study, we investigated a prey-preservation mechanism in a solitary ground nesting wasp, the European Beewolf *Philanthus triangulum* F. (Hymenoptera: Crabronidae; formerly Sphecidae).

Females of the European beewolf hunt and paralyze exclusively honeybee workers (*Apis mellifera*) and deposit one to six prey in a brood cell as food for a larva (Strohm, 1995, 2000; Herzner et al., 2005). The larva hatches 2–3 d after oviposition and feeds on the bees for another 5–8 d. Under the humid and warm conditions in the brood cell, the nutrient-rich provisions are susceptible to spoilage due to microbial degradation. Surprisingly, in the field, only about 5% of brood cells are seriously infected with fungi (Strohm and Linsenmair, 2001). This low rate can be attributed to a treatment of the prey by beewolf females that delays the onset of fungal growth (Strohm and Linsenmair, 2001). Beewolf females extensively lick the whole surface of the paralyzed prey before oviposition. Strohm and Linsenmair (2001) hypothesized that, during this licking, females apply an antifungal secretion from large postpharyngeal glands (PPG; Strohm et al., 2007) to the surface of the honeybees. Here, we compared the composition of the PPG content and the surface of provisioned honeybees from brood cells by using combined gas chromatography-mass spectrometry (GC-MS). We tested also for an antifungal effect of the PPG secretion by using inhibition-zone assays.

Methods and Materials

Beewolves Beewolf females were obtained either from a field population close to the Biocenter of the University of Würzburg, Germany, or from a laboratory population kept at the University of Würzburg (daughters of field-caught females). They were housed individually in observation cages in a climate chamber (26/22°C day/night 14/10 hr light/dark cycle) and provided with honeybees as prey and honey *ad libitum*. The observation cages allow the withdrawal of paralyzed bees immediately after paralyzation and the removal of regularly provisioned bees from brood cells shortly after oviposition. A detailed description of the observation cages is given in Strohm and Linsenmair (1994/1995).

Postpharyngeal Secretion and Cuticular Extracts of Provisioned and Paralyzed Bees To obtain PPG extracts for chemical analysis, glands were removed from the heads of freshly killed beewolf females (N=5) by grasping the hypopharynx with tweezers and gently pulling the attached gland out through the mouth. The glands were immediately transferred to 250 µl distilled *n*-hexane (Fluka, Deisendorf, Germany) and extracted for 1 hr.

To test whether chemical compounds are applied to the surface of the honeybees during the licking by beewolf females and to test whether these are derived from the PPG, we extracted honeybees that were removed from females within a few minutes after paralyzation (paralyzed bees, N=6) and honeybees from brood cells that had been regularly provisioned and licked (provisioned bees, N=6) by females. All bees were taken from the

same stock population of the Department of Animal Physiology and Sociobiology, University of Würzburg. Analyses of provisioned and paralyzed bees were done at the same time during the season. The bees were extracted individually for 10 min in 500 µl distilled *n*-hexane (Fluka, Deisendorf, Germany). A known amount of an internal standard (octadecane) was added to each extract for quantification.

All samples were reduced in volume to approximately 100 µl by a gentle stream of nitrogen. An aliquot of 1 µl of each sample solution was analyzed by GC-MS.

Capillary Gas Chromatography-Mass Spectrometry GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 mass selective detector. The GC was equipped with an RH-5ms+ fused silica capillary column (30 m×0.25 mm ID; film thickness=0.25 µm). The temperature program used was 60°C for 1 min, then to 300°C at 5°C min^{-1} and held for 10 min. A splitless injector (250°C) was used with helium, at a constant flow of 1 ml min^{-1}, as the carrier gas. Electron ionization mass spectra (EI-MS) were recorded at 70 eV.

The chemical composition of PPG content and cuticular hydrocarbons of honeybees have been determined in other studies and are described elsewhere in detail (Schmitt et al., 2007; Strohm et al., unpublished data). In earlier analyses, we used different solvents and solid phase microextraction to obtain a full inventory of all compounds. These analyses showed that hexane extracts gave representative profiles of the compounds present. In the present study, *n*-alkanes were identified by comparing retention times and mass spectra of the extracts with data from the earlier analyses and with data from a commercial MS library (NIST, Gathersburg, MD, U.S.A.). Alkenes were identified by mass spectra and their retention times. The position and configuration of double bonds were inferred by comparison with earlier analyses on the chemistry of the PPG and cuticular hydrocarbons of beewolf females that used Fourier transform infrared (FTIR) spectrometry and dimethyl disulfide (DMDS) derivatization (Strohm et al., unpublished data); peaks with identical retention times were considered to be the same isomers. Some alkenes were present only at trace amounts and could not be characterized completely. Alkadienes were identified by mass spectra and retention times. Due to the small amounts of these compounds, the position and configuration of the double bonds were not determined. Methyl alkanes were identified by diagnostic ions (Blomquist et al., 1987) and by determining Kovats indices according to the method of Carlson et al. (1998). The ketones, Δ-16-pentacosen-8-one and Δ-18-heptacosen-10-one, were identified by comparing retention times and mass spectra with data from our earlier analyses (Strohm et al., unpublished data). Some chemicals that were identified in our previous analyses of the PPG, by using pooled extracts of several females, were not detected in extracts of single females analyzed in this study.

Peak areas were obtained by manual integration, and the amounts of compounds were calculated by comparing peak areas to the internal standard. Some peaks were not separated satisfactorily and were treated as one peak for integration (see Table 1). In particular, (*Z*)-9- and (*Z*)-7-alkene isomers and corresponding alkadienes were generally not resolved. The (*Z*)-9-alkene was always the most dominant peak among the three.

Data Analysis For each individual bee extract, the total peak area was standardized to 100%, and a multivariate analysis was performed to assess the similarity or divergence of the chemical profiles of paralyzed honeybees, provisioned honeybees, and the PPG secretions. Because peak areas represent compositional data, they were transformed to logcontrasts (Aitchison, 1986; Reyment, 1989) before analysis. However, the original transformation procedure makes it necessary to exclude compounds that do not occur in all

samples. We, thus, modified the transformation (log(relative peak area+0.1/(geometric mean of all peak areas+0.1))) to avoid undefined values for peaks with an area of zero.

The transformed relative peak areas were subjected to a *principal component analysis (PCA)* to reduce the number of describing variables. The extracted PCA factors were subjected to a *discriminant analysis (DA)* to assess whether the three different groups (paralyzed bees, provisioned bees, PPG secretions) could be separated on the basis of their chemical profile.

To assess the pattern of similarity between paralyzed bees, provisioned bees, and PPG secretions without an *a priori* grouping (unlike in the DA), we performed a *hierarchical cluster analysis*. We used the squared Euclidean distance as a measure of distance for cluster analysis and within groups average linkage as the method for combining clusters (other methods of combining clusters yielded qualitatively identical results).

The overall amounts of cuticular compounds on provisioned and paralyzed bees were compared with an exact test for independent samples. All analyses were calculated by using SPSS 13.0.

Antifungal Activity of the Postpharyngeal Gland Secretion Activity of the PPG secretion against fungal growth was measured by an inhibition-zone assay (see e.g., Gross et al., 1998, 2002). An extract of provisioned bees was tested and compared against a known antifungal compound (see below). The extract of provisioned bees was obtained by soaking 75 provisioned bees in distilled hexane twice for 10 min. Extracts were combined, and the amounts of hydrocarbons were determined by GC-MS analysis as above.

The test fungi used were (1): the predominant, naturally occurring fungus in original brood cells (not yet identified fungus of the genus *Aspergillus*) taken directly from an infested bee in a brood cell ("brood cell fungus"), (2): *Aspergillus fumigatus* (Deutsche Sammlung von Mikroorganismen und Zellkulturen, # DSM 819), and (3): *Penicillium roquefortii* (Department of Hygiene, University Hospital of Würzburg, # V 308). Fungal spores were obtained from solid medium cultures and suspended in liquid medium for inoculation. Agar plates (9 cm diameter) were prepared with a standard fungus medium (4 g yeast, 10 g maltose, 12 g agar, 8 ml 50% glucose solution, and 992 ml distilled water). For inoculation, 10 µl of the spore suspension were evenly spread over the sterile agar surface.

The test chemicals or secretions were applied to disks of sterile filter paper (5 mm diameter), which were then placed on the agar surface. Different amounts of extracted hydrocarbons from licked honeybees, and the monoterpenoid citral, were tested. Citral is a known antifungal agent produced by different arthropods (Cole et al., 1975; Okamoto et al., 1981; Cane et al., 1983) and was applied in the same amounts as the PPG secretion to allow for a comparison of the effect. We chose 160 µg as the maximum amount tested because this was the maximum amount of hydrocarbons found on a single bee in our analyses. To each filter paper disk, we applied 10 µl of hexane containing different amounts of hydrocarbons extracted from provisioned bees (a: 20 µg, b: 40 µg, c: 80 µg, d: 160 µg) or different amounts of citral (e: 20 µg f: 40 µg, g: 80 µg, h: 160 µg, Fluka, Deisendorf, Germany). The control (i) was treated with *n*-hexane. Each Petri dish received nine differently treated (solutions a–i) filter paper disks.

Tests were replicated six times for the brood cell fungus and *P. roquefortii* and five times for *A. fumigatus*. After a 3-d incubation of the parafilm-sealed agar plates at 26–29°C, the diameters of the halos in fungal mats, caused by the antifungal action of the samples, were measured. We tested for a dose–effect relationship by conducting regression analyses.

Results

PPG Secretion and Cuticular Extracts of Paralyzed and Provisioned Bees Typical gas chromatograms of hexane extracts of paralyzed honeybees and provisioned honeybees and the content of the PPG of *P. triangulum* females (Fig. 1), showed a qualitative concordance (see also Table 1). Of the total of 52 substances detected, 42 occurred in all three types

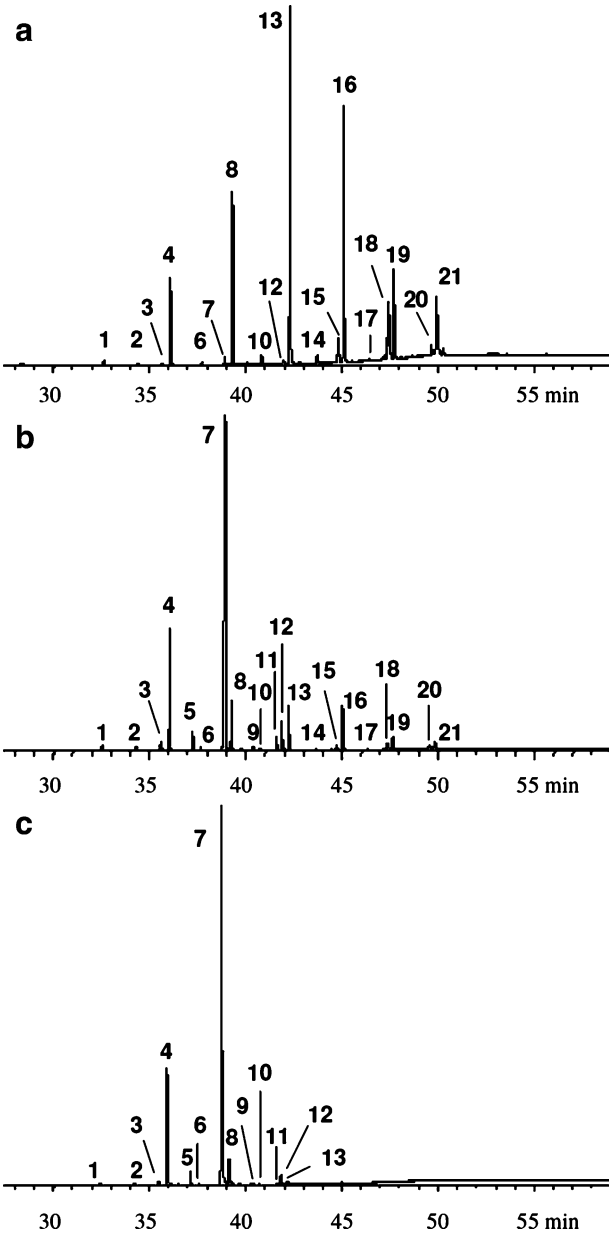


Fig. 1 Gas chromatograms of hexane extracts of **a** a paralyzed but not provisioned honeybee (*A. mellifera*), **b** a honeybee provisioned by a beewolf female, and **c** a postpharyngeal gland of a beewolf female (*P. triangulum*). The peaks of minor components are not visible due to the scale used. The peaks correspond to the following substances: *1* heneicosane; *2* docosane; *3* Δx,y-tricosadiene, (*Z*)-9-tricosene, (*Z*)-7-tricosene; *4* tricosane; *5* (*Z*)-9-tetracosene; *6* tetracosane; *7* (*Z*)-9-pentacosene, (*Z*)-7-pentacosene; *8* pentacosane; *9* (*Z*)-9-hexacosene; *10* hexacosane; *11* Δ-16-pentacosen-8-one; *12* (*Z*)-9-heptacosene, (*Z*)-7-heptacosene; *13* heptacosane; *14* octacosane; *15* (*Z*)-9-nonacosene; *16* nonacosane; *17* triacontane, *18* Δx,y-hentriacontadiene, (*Z*)-9-hentriacontene, (*Z*)-7-hentriacontene; *19* hentriacontane; *20* Δx,y-tritriacontadiene, Δx-tritriacontene; *21* tritriacontane (the chromatograms were obtained from extracts not containing the internal standard)

of extracts. Δx-heineicosene, Δx-docosene, (*Z*)-9-hexacosene, and the ketones, Δ-16-pentacosen-8-one and Δ-18-heptacosen-8-one, could be detected only in the PPG and on provisioned bees. The methyl-hentriacontanes and Δx-dotriacontene were not detected on provisioned bees. The PPG extracts contained no detectable amounts of Δx-dotriacontene and tritriacontane.

Although the cuticular chemicals found in extracts of paralyzed and provisioned bees were qualitatively similar, there were pronounced quantitative differences between them

Table 1 Comparison of the relative amounts of cuticular substances[a] on paralyzed and provisioned bees[b]

Compound	Paralyzed Bees	Provisioned Bees	Present in PPG?[c]
Δx-Nonadecene	0.03±0.07	0.05±0.03	+
Nonadecane	0.06±0.04	0.02±0.02	+
Δx-Heneicosene	–	0.01±0.01	+
Heneicosane	0.55±0.26	0.45±0.11	+
Δx-Docosene	–	0.01±0.01	+
Docosane	0.10±0.13	0.32±0.03	+
Δx,y-Tricosadiene,(*Z*)-9-Tricosene,(*Z*)-7-Tricosene	0.35±0.44	1.42±0.52	+
Tricosane	5.21±3.46	15.65±2.83	+
Methyl tricosanes (11-, 9-, 7-, 5-)	0.05±0.08	0.12±0.02	+
(*Z*)-9-Tetracosene	0.02±0.06	1.90±0.17	+
Tetracosane	0.15±0.11	0.34±0.09	+
Δx,y-Pentacosadiene, (*Z*)-9-Pentacosene,(*Z*)-7-Pentacosene	1.74±3.03	56.56±4.99	+
Pentacosane	7.95±2.97	7.18±3.25	+
Methyl pentacosane (11-, 9-, 7-, 5-)	0.41±0.25	0.15±0.03	+
(*Z*)-9-Hexacosene	–	0.30±0.07	+
Hexacosane	0.37±0.10	0.18±0.05	+
Δ-16-Pentacosen-8-one	–	1.61±0.49	+
(*Z*)-9-Heptacosene,(*Z*)-7-Heptacosene	0.61±0.50	2.47±0.33	+
Heptacosane	17.38±6.73	4.36±1.59	+
Methyl heptacosane (13-, 11-, 9-)	1.20±0.73	0.06±0.02	+
Octacosane	0.46±0.10	0.11±0.07	+
Δ-18-Heptacosen-10-one	–	0.06±0.02	+
(*Z*)-9-Nonacosene	1.66±0.51	0.39±0.29	+
Nonacosane	15.86±1.69	2.92±1.68	+
Methyl nonacosane (15-, 13-, 11-)	0.88±0.49	0.03±0.01	+
Triacontane	0.17±0.15	0.04±0.02	+
Δx,y-Hentriacontadiene, (*Z*)-9-Hentriacontene, (*Z*)-7-Hentriacontene	15.19±3.82	1.19±0.77	+
Hentriacontane	9.07±2.88	1.08±0.50	+
Methyl hentriacontane (15-, 13-, 11-)	0.71±0.27	–	+
Δx-Dotriacontene	0.61±0.52	–	−
Δx,y-Tritriacontadiene	1.97±0.84	0.17±0.15	+
Δx-Tritriacontene	15.90±5.99	0.78±0.37	+
Tritriacontane	1.34±0.53	0.09±0.05	−

The last column indicates whether the substance was present in at least one of the PPG samples.

[a] Mean ± standard deviation

[b] $N=6$

[c] + Yes, − no

(Fig. 1 and Table 1). On average, the total amount of cuticular compounds was about five times higher on provisioned bees (109.9±28.2 μg) compared to paralyzed bees (23.2±5.0 μg; exact P=0.002, two-tailed). The cuticular profile of paralyzed bees was dominated by the saturated hydrocarbons, heptacosane, nonacosane, and pentacosane. On the cuticle of provisioned bees, the unsaturated hydrocarbon (*Z*)-9-pentacosene was by far the most abundant compound. (*Z*)-9-Pentacosene was also the predominant compound in four of the five PPG extracts. In PPG extract 4 (*Z*)-9-heptacosene was the major compound.

To analyze statistically the similarity or discrepancy of the chemical profiles of paralyzed bees, provisioned bees, and PPG secretions, multivariate data analysis was performed. The two principal components produced by the *PCA*, explained 70.3% of the total variance. A *DA* on these principal components significantly differentiated extracts of paralyzed bees, provisioned bees, and PPGs (Wilks' λ=0.018, χ^2=54.4, df=4, P<0.001, Fig. 2). Discriminant function 1 explained 98.9% of the total variation extracted by the *PCA*, and discriminant function 2, only 1.1%. By far, the largest effect was between paralyzed bees versus provisioned bees and PPG secretions.

The *cluster analysis* (Fig. 3) separated paralyzed bees from both provisioned bees and PPGs in the first bifurcation. The distinction was less pronounced between provisioned bees and PPG secretions. The second bifurcation separated PPG extract 4 from all other PPG extracts, and the provisioned bees, and thus, placed it in a unique branch. The third bifurcation separated provisioned bees from PPGs, with the exception of provisioned bee 1, which clustered within the PPG extracts. According to this analysis, provisioned bees are very similar to PPG content but dissimilar from paralyzed bees. Furthermore, the amount of hydrocarbons on provisioned bees was considerably increased. Thus, beewolf females seem to apply the secretion of their PPG to the surface of their prey before oviposition.

Antifungal Activity of the Postpharyngeal Gland Secretion The diameters of the inhibition zones are given in Table 2. Hexane had no fungistatic effect on any of the fungi (except for one disk in an *A. fumigatus* Petri dish that showed an inhibition zone of about 1.4 mm). The extract of provisioned bees showed no effect on growth of any of the fungi at any concentration in the inhibition-zone assay. Not only did the extract fail to produce inhibition

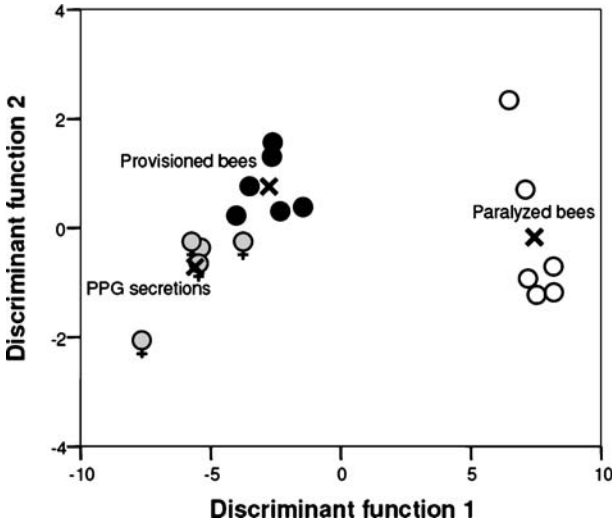


Fig. 2 Plot of the two discriminant functions obtained by the discriminant analysis based on the relative peak areas of extracts from paralyzed honeybees (N=6, *open circles*), provisioned honeybees (N=6, *closed circles*), and postpharyngeal glands of *P. triangulum* females (N=5, *grey symbols*) (*x* Group means; see text for details)

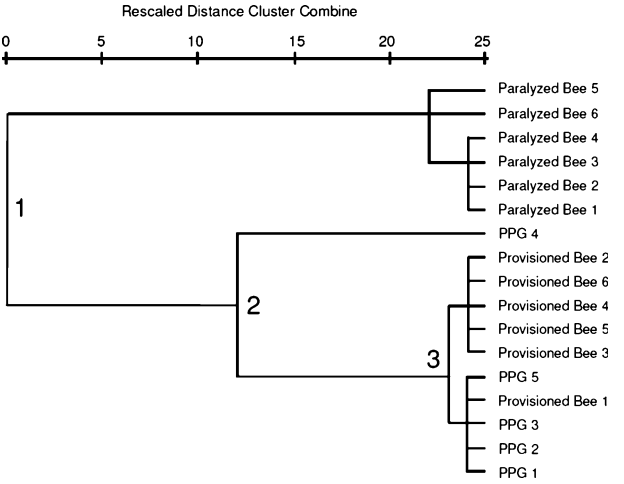


Fig. 3 Dendrogram based on the cluster analysis of cuticular compounds of individual extracts of paralyzed bees, provisioned bees, and postpharyngeal glands of *P. triangulum* females (numbers indicate the different individuals). Numbers in the dendrogram indicate the first three bifurcations (see text)

zones, but fungi grew even on the filter paper disks. Citral was active against *A. fumigatus*, causing clear halos, but not against the brood cell fungus and *P. roquefortii* (Table 2). For citral, we found a significant positive dose–effect relationship on *A. fumigatus* (r=0.87, P< 0.001, N=25). There was no such effect for provisioned bee extract (r=0.11, P=0.30, N=25). A comparison of the two regression lines (citral: y=0.0569x + 1.3158, provisioned bee extract: y=−0.0011x + 0.1267) showed that the slopes were highly significantly different (Z=8.754, P<0.001).

Discussion

We showed that provisioned bees carry roughly five times more hydrocarbons than paralyzed bees and that the cuticular chemical profile of provisioned prey bees is similar to

Table 2 The effect of provisioned bee extract, citral, and hexane (Control) [mean halo diameter (mm) ± standard deviation] on the three tested fungi

Test solutions	Brood cell fungus (*Aspergillus*)[a]	*A. fumigatus*[b]	*P. roquefortii*[a]
Extract of provisioned bees			
20 μg	0	0	0
40 μg	0	0	0
80 μg	0	0	0
160 μg	0	0	0
Citral			
20 μg	0	3.81±0.98	0
40 μg	0	3.75±1.00	0
80 μg	0	4.83±1.87	0
160 μg	0	10.47±3.08	0
Hexane			
Control	0	0.27±0.61	0

[a] N=6

[b] N=5

the content of the PPG of beewolf females. The two ketones, Δ-16-pentacosen-8-one and Δ-18-heptacosen-8-one, were present only in the PPG of beewolf females and on provisioned bees and were not present on paralyzed bees. These results, together with the behavioral observations of an extensive licking of the surface of prey bees by beewolf females (Strohm and Linsenmair, 2001), provide evidence that females apply large amounts of PPG secretion to the surface of paralyzed honeybee prey during the licking treatment.

PPG extract number 4 was separated in the cluster analysis from all other PPG secretions and was placed in a unique branch. PPG content of female *P. triangulum* shows a marked dimorphism: the predominant compound in the hydrocarbon mixture is either (*Z*)-9-pentacosene, or less frequently, (*Z*)-9-heptacosene (Strohm et al., unpublished data). Female number 4 was of the heptacosene type, whereas all others were of the pentacosene type. All provisioned bees were randomly selected, but turned out to be of the pentacosene type. Provisioned bee number 1 clustered within the PPG secretions of the pentacosene type, probably because this bee carried larger amounts of the secretion (160 μg) than all other bees (mean: 109.9±28.2 μg) and was, thus, more similar to the pure PPG secretion.

Previous studies have shown that the licking treatment of prey by female beewolves delays fungus growth on prey bees (Strohm and Linsenmair, 2001; Herzner and Strohm, 2007). The results of the present study suggest that this effect is not due to direct chemical (toxic) activity of the PPG secretion against fungi. Even 160 μg of hydrocarbons, corresponding to the maximal amount found on a single provisioned bee in our study, on a 5-mm filter paper disk failed to impede fungal growth in our assays. The surface area of the honeybee, including its hairs, setae, and sensilla, is larger than the surface of the filter paper disk. Therefore, the concentration of secretion on provisioned bees is likely to be considerably lower than that used on the disks in our tests.

Citral was active against *A. fumigatus*, but not against the other two fungi. The antifungal effect of citral, a component of mite alarm pheromones, on *A. fumigatus* was previously reported by Okamoto et al. (1981). Citral has also proven to be active against several other fungi and bacteria (Cole et al., 1975; Cane et al., 1983). A similar selectivity against microorganisms has been observed previously for some other insect-derived substances or secretions (Cole et al., 1975; Sosa-Gomez et al., 1997; Bot et al., 2002; Gross et al., 2002).

Inhibitory effects of exocrine gland secretions on microorganisms have been reported from a variety of insect species (e.g., solitary bees: Cane et al., 1983; ants: Cole et al., 1975, Bot et al., 2002; leaf beetles: Gross et al., 2002, stink bugs: Sosa-Gomez et al., 1997). Usually, these secretions have a direct antimicrobial effect. That the observed delay in growth of fungus on provisioned bees (Strohm and Linsenmair, 2001; Herzner and Strohm, 2007) does not appear to be mediated by a direct antifungal effect of the PPG secretion is thus unusual. The PPG secretion comprises predominantly hydrocarbons, a class of chemicals that is not expected to have antimicrobial effects. Therefore, how does the licking treatment and the PPG secretion interfere with mold fungi on the prey bees?

First, by licking the surface of the bees, beewolf females might clean the surface and remove fungal spores. Second, the treatment could change physico-chemical properties of the surface. The cuticle is the first contact between host and fungal conidia, and it is plausible that physico-chemical characteristics of the cuticle influence conidial adhesion and germination and fungal proliferation. The attachment of fungal propagules to host cuticle is mediated by chemical components on the epicuticle (Fargues, 1984; Boucias et al., 1988; Boucias and Pendland, 1991; Sosa-Gomez et al., 1997; Pavlukova et al., 1998). In the southern green stink bug, *Nezara viridula*, for example, the removal of hydrocarbons caused a significant reduction of conidial binding to the cuticle (Sosa-Gomez et al., 1997).

The hydrocarbons extracted (with hexane) from *N. viridula* exuvia, and applied to glass substrates, allowed the attachment of conidia, suggesting that hydrocarbons play an important role in this first step of infection. The PPG treatment changes the hydrocarbon profile of the bees considerably and might, therefore, alter or mask particular physico-chemical characteristics of the cuticle (e.g., hydrophobicity or charge) that are necessary for adhesion of conidia (Sosa-Gomez et al., 1997; Fletcher et al., 1980).

Third, the treatment could affect the next step in the infection process of fungi, conidial germination. Germination often requires exogenous carbon sources; chitin and other cuticular constituents have been shown to serve this purpose (Smith and Grula, 1981; Napolitano and Juárez, 1997; Crespo et al., 2002). The secretion from the PPG might act as a barrier to the bee surface, preventing conidia from coming into contact with nutrients or other cues that could trigger germination. Finally, the germination of conidia also requires sufficient water. In a related study, we showed that the PPG secretion considerably reduces water condensation on the surface of prey (Herzner and Strohm, 2007), thereby indirectly impairing fungal germination. Therefore, hydrocarbons from the PPG, applied in large amounts to the bees' surface, mediate a protective effect by an indirect, physical, rather than a direct, chemical mechanism.

Acknowledgements We thank the Department of Behavioral Physiology and Sociobiology, Würzburg University, for providing honeybees, and Sigrid Lempert, Department of Hygiene, Würzburg University, for providing fungus cultures. This study was partly supported by the DFG (SFB 554, TP B3, STR 532/1-2).

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J Chem Ecol (2007) 33:861–869
DOI 10.1007/s10886-007-9262-9

Species-specific Expression of Major Urinary Proteins in the House Mice (*Mus musculus musculus* and *Mus musculus domesticus*)

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Received: 25 June 2004 / Revised: 22 November 2006 / Accepted: 1 December 2006 /
Published online: 28 February 2007
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Abstract The analysis of expression of pheromone-carrying major urinary proteins (MUPs) from two subspecies of house mice (*Mus m. musculus*, *Mus m. domesticus*) was studied. It has been previously shown that commensal populations of the two subspecies can discriminate on the basis of urinary signals. MUPs are predominant urinary proteins that protect pheromones from rapid degradation in a hydrophilic environment, and individuals of *M. m. musculus* tend to rely on these urinary cues in the process of subspecies discrimination more than *M. m. domesticus* individuals. Although it is not precisely known what triggers phenotypic and epigenetic changes of MUP expression, our results show that in the subspecies *M. m. musculus*, sex is a significant factor influencing variations in the regulation of selected MUPs in the liver. Furthermore, male *M. m. musculus* individuals expressed all the studied MUPs' mRNA significantly more than females or individuals of either sex in *M. m. domesticus*. Correspondingly, the pattern of mRNA abundance was corroborated with the level of total MUP concentration in the urine, such that the level of sexual dimorphism was also significant and species-specific. Our finding introduces a hypothesis that quantitative variation of these proteins may be an essential part of a subspecies recognition system that maintains homospecific mixing.

Keywords Gene expression · MUP · Sexual dimorphism · *Mus*

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Introduction

The urine of *M. musculus* Linnaeus, 1758 contains large quantities of acidic protein isoforms called major urinary proteins (MUPs) with a molecular mass 18–19 kDa (Cavaggioni and Mucignat-Caretta, 2000). These proteins are synthesized in the liver and released through the kidney into the urine. Although, the production of MUPs indicates that it must have advantages, little is known about the constraints that have shaped the evolution of expression differences of this protein family. In *M. m. domesticus*, Schwartz & Schwartz, 1943, it has been repeatedly shown that MUPs carry volatile nonpolar pheromones in their hydrophobic pocket (Bacchini et al., 1992; Žídek et al., 1999). The combination of volatile pheromones and their protein carriers may facilitate individual/status recognition and estrus synchronization through a slow release of these ligands from proteins when the urine mark is deposited (Hurst et al., 1998, 2001; Novotny et al., 1999; Marchlewska-Koj et al., 2000). Therefore, these proteins are advantageous for an individual, as the essential information on sex and status is present even in the absence of the territory owner (Hurst et al., 2001). In addition to the information on genetic relatedness that is manifested by the highly polymorphic major histocompatibility complex (MHC), MUPs are important in their ability to reveal temporal individual-status information. If common androgen regulators correlate with the temporal individual status, they may also constitute a method of honest signaling.

One of the remarkable characteristics that have emerged from the study of MUPs is their diversity. This variation is largely caused by single nucleotide and/or single codon mutations (Robertson et al., 1996; Beynon et al., 2002). MUPs are the product of a multigene family of approximately 30 genes and pseudogenes located on chromosome 4 (Bishop et al., 1982). Their expression is largely sex-dependent, such that males produce more than females do (Wicks, 1941), with the pattern and amount probably regulated by testosterone, growth hormone, and thyroid hormone levels (Knopf et al., 1983). For example, females injected with testosterone produce amounts of MUPs similar to those of their male counterparts (Cavaggioni and Mucignat-Caretta, 2000). Sequence variations and different expression patterns have been reported for various laboratory strains (Finlayson et al., 1965), and it is likely that these differences represent polymorphisms that have been detected in wild populations (Pes et al., 1999; Robertson et al., 1997). What has yet to be determined is the species-specific expression pattern of MUPs in wild mice of the genus *Mus* and to what extent these "expression profiles" may influence or represent a signal for either species recognition and/or a sexually selected behavior. Such testing may further the understanding of natural hybridization between the two subspecies under study.

To further elucidate whether major urinary proteins in mice are a product of sexual selection, we tested the hypothesis that differences between male and female expression patterns are greater than differences between the two subspecies. Sampsell and Held (1985) discovered—with an application of a technique known as dot-blot—little variation in MUP mRNA quantity between male and female *M. m. domesticus* but larger differences in *M. m. musculus*. Our study aimed for a more precise quantification of the expression variation in wild caught mice of both subspecies. Our model organisms, *M. m. musculus* and *M. m. domesticus*, recolonized Europe after glaciation and formed a narrow hybrid zone about 20 km wide spanning across Europe from Denmark to Bulgaria. Regarding their ecology, populations of both subspecies live commensally (i.e., syn-anthropically), and to date no published data suggest that there are significant ecological differences between them (Boursot et al., 1993). The rationale for testing this hypothesis is highlighted by the fact that it would be advantageous for an individual to recognize individuals of the same species and those of another to avoid potential problems with F1 hybrid sterility. One can

envisage two likely, yet understudied, scenarios: (1) MUP quantity and expression pattern are a product of sexual selection and, therefore, females choose males on the basis of their MUP-related smell, regardless of the species, or (2) MUP signals are a trait under purifying selection and form an efficient species-specific barrier to diminish the cost of producing sterile litters. The former scenario could partially explain an introgression of several *domesticus* genes into *musculus* populations (Munclinger et al., 2002) as a consequence of such a recognition error, whereas the latter scenario may partially explain why the narrow hybrid zone is relatively stable. Our approach in analyzing MUP expression profiles combines real-time polymerase chain reaction (PCR) of unique mRNA/cDNA sequences and densitometry analysis of isolated urinary MUPs run on polyacrylamide gel electrophoresis under denaturating condition (i.e., SDS-PAGE).

Methods and Materials

Subjects Eighty-three reproductively active (approximately 70-d-old) mice were used. Different mice were used for the isolation of mRNA (29 individuals, i.e., *M. m. domesticus*—eight males + seven females, *M. m. musculus*—eight males + six females) and for the urine collection (54 individuals, i.e., *M. m. domesticus*—12 males+14 females, *M. m. musculus*—15 males+13 females). The mice were trapped at three *domesticus* sites in Germany (i.e., Straas, Neunreuth, and Wuppertall), and at six *musculus* sites in the Czech Republic (i.e., Sedlecko, Vintirov, Praha, Brandys, Studenec, and Svetla Hora).

RNA Isolation and cDNA Synthesis Total RNA from a 50-mg liver tissue was extracted with ToRNAzol reagent (GeneAge Technologies, a.s., Prague, Czech Republic) according to the manufacturer's protocol. The purity of RNA was assessed from the ratio of the optical densities at 260 and 280 nm, and the integrity was controlled by electrophoresis on 1% agarose gel containing ethidium bromide.

Five micrograms of total RNA were used for synthesis of single-stranded cDNA according to a first-strand cDNA synthesis protocol (Fermentas UAB, Vilnius, Lithuania) with RevertAid™ M-MulV Reverse Transcriptase and oligo$(dT)_{18}$ primer. To minimize variation in the reverse transcription, all RNA samples were reverse-transcribed simultaneously.

Table 1 A set of specific primers that were used for the real-time amplification of given products

UniSTS Name	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size (bp)
MUP1	AATGCCAATCGCTGCC	GCTCCGAATTATCTATGGTTGC	430
MUP2	ATTAATGGGGAATGGCATACTA	GGATTCCATGCTCCTCACAT	388
MUP3	GCAAGTGTAATCATTTATTGAACAGG	ATGGAGCTCTATGGCCGAG	407
MUP4	TTCCGATCGATACAGCATTG	TTGAACAGGAAGAGGAAGCAA	189
MUP5	TGGCACCATGAGAGTTTTTG	CACTGGAGGCTCAAACCATT	404
Beta 2-microglobulin	AGTCTTTCTGGTGCTTGTCTCA	TATCAGTCTCAGTGGGGGTGA	248
HPRT*	CCTCATGGACTGATTATGGACA	AGTTGAGAGATCATCTCCACCA	239

*Hypoxanthine phosphoribosyltransferase

Quantitative Real-Time PCR The resultant cDNA was amplified with PCR by using primers given in Table 1. The target sequences belong to the six most abundant MUP variants in mice (Shahan et al., 1987). Amplified PCR products were consequently sequenced to ensure correct priming with the target sequences of given MUPs. Real-time PCR was performed in a Rotor-Gene 2000 from Corbett Research by using the SYBR® Green detection method. Total reaction volume was 25 μl of buffer solution containing 1 μl of cDNA sample, 3 mM $MgCl_2$, 0.2 mM dNTP, SYBR® Green 1: 25,000, 0.4 U/μl taq polymerase, and 0.2 μM primers. PCR amplifications were always performed in duplicate wells under the following conditions: 2 min at 95°C, then 30 repetitive cycles: 30 sec at 95°C, 30 sec at 56°C, 30 sec at 72°C, and an additional fourth step of 15 sec at 82°C where the fluorescent data were collected to avoid any nonspecific signals derived by eventual primer dimers or unspecific minor product (Pfaffl, 2003). This fourth step ensures accurate quantification of desired product, and the appropriate product compound is checked in all samples by the melting analysis. The beta-2-microglobulin, out of five tested housekeeping genes, was assayed as a normalization control to correct any loading discrepancies (Bustin, 2000; 2002), and hypoxanthine phosphoribosyltransferase (HPRT) was used as a control gene for each individual. Control reactions with "RT minus template" did not produce significant amplification product (Vandesompele et al., 2002).

Relative quantitation of target gene expression was evaluated by using the comparative Ct method (Giulietti et al., 2001) by determining the cycle threshold (Ct) based on the fluorescence detected within the geometric region of the semilog view of the amplification plot. The ΔCt value was determined by subtracting the target Ct of each sample from its respective beta-2-microglobulin Ct value. Calculation of ΔΔCt involves using the highest sample ΔCt value as an arbitrary constant to subtract from all other ΔCt sample values. Fold changes in gene expression of the target gene are equivalent to $2^{-\Delta\Delta_{Ct}}$ (Livak and Schmittgen, 2001). Relative fold change (i.e., a change in an order of magnitude), therefore, represents an exponent value, whereas the threshold value of the amplification cycle (i.e., Ct) ensures that the levels are measured at a linear range.

Protein Analysis Urine was collected by a gentle bladder massage and frozen at −20°C. The volume of 0.2 μl of male and 0.4 μl of female urine, respectively, was used for protein analysis. SDS-polyacrylamide gel electrophoresis was performed on Mini-PROTEAN III Electrophoresis System (BioRad Laboratories, Hercules, CA, USA) according to modified protocol by Laemmli (1970). To avoid potential errors caused by a loading inaccuracy, we used Carbonic Anhydrase (29 kDa) marker for SDS-PAGE (Sigma-Aldrich, St. Louis, MO, USA) as standard. Thirteen microliters of sample buffer (containing 1,000 ng of carbonic anhydrase) were added to each sample. The gels were stained with Coomassie® G-250 (SimplyBlue™ Safe Stain, Invitrogen Life Technologies, Paisley, UK). The gel images were acquired by using Gel Doc XRS, and Quantity One 1-D Analysis Software (BioRad Laboratories, Hercules, CA, USA) was used for the densitometry analysis. The total urinary MUP concentrations were calculated from the adjusted volumes that were obtained after corrections by using carbonic anhydrase of known volumes as internal standards.

Statistical Analysis As the mRNA expression and protein concentration were collected from different individuals, we performed two separate analyses. General Linear Model (GLM-Factorial ANOVA) was used to test for dependences on two categorical variables (sex and species). Two separate models were used for detecting differences on the level of mRNA abundance or the level of protein.

Results

Quantitative analysis of mRNA expression by using real-time PCR techniques traditionally employs reference or housekeeping genes to control any errors between samples. To ensure the robustness of our housekeeping system of normalization, we used two reference genes. Beta-2-microglobulin was used as a primary housekeeping gene for this system (i.e., housekeeping by which all other genes including second housekeeping—HPRT—were normalized). Main effects ANOVA revealed that none of the tested factors (i.e., species, individual, sex) had significant effects on the variation of the mRNA abundance of a second housekeeping gene HPRT that was normalized by the first one (MANOVA, factor sex: F=0.931, df=1, P=0.34; species F=0.59, df=1, P=0.45; sex × species F=0.6, df=1, P=0.43). Thus, the following expression variations represent biologically relevant differences.

Predominant urinary MUPs, originally described as MUP1, 2, 3, and 5, are abundant in the liver in both *M. m. musculus* and *M. m. domesticus*. The factorial MANOVA revealed that the major source of variation in mRNA abundance is influenced by sex (F=8.38, df=1, P=0.005), sex × species (F=6.02, df=1, P=0.016), species (F=5.55, df=1, P=0.02), and also marginally by genes (F=2.8, df=3, P=0.042) because of a lower activity of MUP5. *Post hoc* Tukey HSD test further revealed that the major source of variation was differences in mRNA abundance between *M. m. musculus* males and females (df=99, P=0.003) as well as differences (df=99, P<0.002) between *M. m. musculus* males and individuals of both sexes belonging to *M. m. domesticus* (Fig. 1). The major result of this analysis, therefore, is the finding that sexual dimorphism in the expression of selected MUP RNAs is species-specific.

Sexual dimorphism and species specificity of MUP expression was further tested by using calibrated densitometry analysis of total MUPs present in the urine. Correct assignment of an ~18-kDa band as a MUP was confirmed by isoelectric focusing and Western blotting (data not shown) using goat anti-mouse MUP serum (YN-GMMUP, Accurate Chemical & Scientific Corporation, Westbury, NY, USA) as a primary antibody. For this antibody, characteristic precipitin lines are obtained against normal mouse urine and purified mouse MUP, and gives a reaction of full intensity. The result of this analysis is depicted in Fig. 2 and demonstrates that the pattern of sexual dimorphism is significant

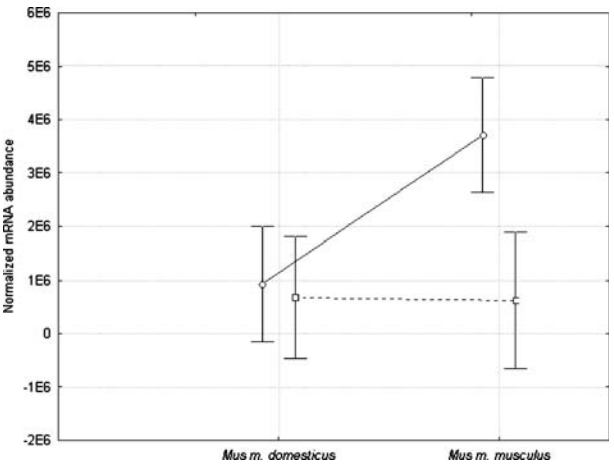


Fig. 1 Subspecies differences in normalized mRNA abundance between *M. m. domesticus* and *M. m. musculus*. Circles connected by a solid line represent differences in mean between males, squares connected by a dashed line represent differences in mean between females with error bars representing confidence intervals (α=0.05)

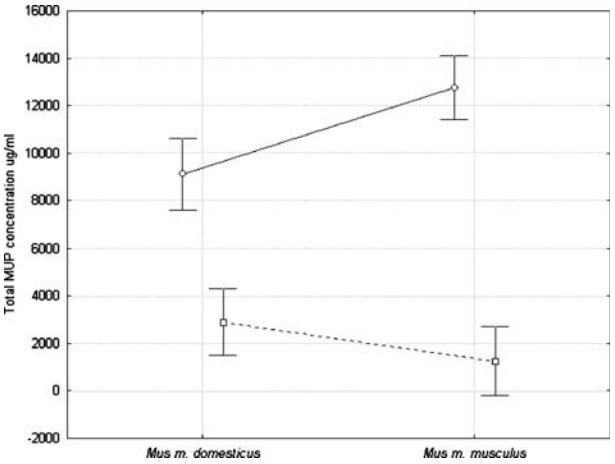


Fig. 2 Subspecies-specific sexual dimorphism in the concentration of major urinary proteins. Circles connected by a solid line represent differences in mean between males, squares connected by a dashed line represent differences in mean between females of both subspecies with error bars representing confidence intervals (α=0.05). It is worth noting that a little difference on the level of mRNA between males and females *M. m. domesticus* (in Fig. 1) yields significant differences on the level of total protein concentration—here demonstrated by nonoverlapping confidence intervals

(F=154.5, df=1, P<0.001) and species-specific (F=13.7, df=1, P<0.001). Our analysis further revealed that MUP production was slightly higher in estrus females, but this trend was not significant (Wilcoxon paired t test: Z=1.153, df=1, P=0.248). However, it is obvious from Fig. 2 that such influence is of limited value as a result of nonoverlapping confidence intervals in each category. It is also worth pointing out that our analysis revealed significant differences between *M. m. domesticus* males and females where other studies have reported no variation (Sampsell and Held, 1985).

Discussion

Differences in gene expression between males and females have been regularly reported in various publications that use high-throughput technologies for arraying large sample sets. Usually, such sexually dimorphic genes are further studied at the level of translation, modifications, and effects on phenotype of studied individuals. The importance of this study is that it shows that species-specific and significant sexual dimorphism at the level of selected MUP mRNA abundance can be corroborated with the level of actual protein concentration in the urine. The strength of this finding is accented by the fact that such species-specific sexual dimorphisms may be a potential mechanism for mate selection differences that have been reported in this species (Smadja and Ganem, 2002).

Our study shows that the abundance of mRNA coding for several major urinary proteins is sexually dimorphic, and that the level of such dimorphism is (sub)species-specific. Major urinary proteins are known to carry volatile pheromones in their hydrophobic beta barrel, and these are released when the urine mark is deposited, and as it slowly dries (Robertson et al., 1993; Hurst et al., 1998). If the intensity of signals directly relates to the abundance of proteins secreted into urine, then this study indicates that individuals of the opposite sex may differentially invest in signaling. Furthermore, we also detected that the level of differences between males and females is smaller in *M. m. domesticus*. This suggests that *M. m. musculus* males invest relatively more energy into MUP production, and that this may potentially be important in the process of female choice. It is also worth noting that our

analysis revealed significant differences in the level of protein concentration and somewhat less reliably (i.e., not significantly) in the level of mRNA between *M. m. domesticus* males and females in which other studies have reported no variation (Sampsell and Held, 1985).

At the behavioral level, Smadja and Ganem (2002) showed that individuals from both populations of the two subspecies can discriminate each other on the basis of urinary signals. However, they also demonstrated that *M. m. musculus* females were choosier than females of *M. m. domesticus*, as when given a choice, they systematically mated with individuals of the same subspecies. Their results, as well as the study by Christophe and Baudoin (1998), corroborate our interpretation of the expression differences detected here, i.e., that males in a more choosy subspecies have to produce more MUPs to outcompete other males in the process of intermale competition and female choice. Moreover, Munclinger and Frynta (1997) lend further support to these findings by revealing that male individuals of *M. m. domesticus* (individuals from Turkey) were less choosy even when given other species (i.e., *M. spretus*, *M. spicilegus*) to choose from. Our findings, along with previously published observations of behavioral discriminations, support the already stated claim that MUPs in *M. m. domesticus* primarily serve to transfer individual specific information (Hurst et al., 2001).

This study deals with the expression differences of MUPs in wild house mice (*Mus musculus*). However, the resulting scent profile is a result of both the combination and interaction of MUPs and pheromones that these proteins carry in their hydrophobic pocket. Recent advances in understanding pheromone communication stem from seminal papers by Novotny (2003) who, following Whitten's definitions (1966) of primer pheromones, demonstrated that two androgen-dependent urinary metabolites, 3,4-dehydro-*exo*-brevicomin and 2-*sec*-butyldihydrothiazole, are effective in promoting estrus synchronization. Other pheromones such as 6-hydroxy-6-methyl-3-heptanone have a profound effect on the acceleration of puberty (Novotny, 2003). An important milestone in the study of pheromonal communication in mice was evidence that MUPs are involved in binding these pheromones (Bacchini et al., 1992) as well as the discovery that nearly all (pheromone) ligands are protein bound (Robertson et al., 1993). To date, there is sufficient evidence in the literature that the action of known and recently discovered pheromones (Novotny, 2003) influence a chain of physiological and behavioral reactions ranging from modulation of estrus to complex behavioral traits such as aggression (Mucignat-Caretta et al., 2004).

Although various published studies explain how scent molecules are transported and protected (e.g., Hurst et al., 1998), there is a need for further determination of which molecules signal identity and which molecules are rather general signals of physiological status specific for each sex. In this respect, our study is important in showing that individual variation in the expression of most of MUPs studied is relatively smaller than that between sexes and species. The abundance of one protein in our study (MUP5) was not species-specific but was highly sexually dimorphic, suggesting that some particular protein isoforms are differentially regulated in each sex regardless of species. The idea of functional specificity for given MUPs is not new (Shahan et al., 1987). However, apart from a few (Pes et al., 1999; Beynon et al., 2002), most studies have been performed on laboratory mice, and species-specificity has not yet been demonstrated in controlled experiments with wild or wild-derived mice. Here, we suggest that because of detected sexual dimorphisms in the abundance of MUP mRNA in the liver and the proteins in the urine, the function of these proteins may rather be sexual in that females use the scent information to evaluate quality of competing males before or during mating. Although, there is no direct evidence that females use marks to evaluate the quality of particular males in the process of mate choice, it is possible that male marking and overmarking (Humphries et al., 1999) may

locally reinforce individual (male) fitness benefits. Thus, the *regulation* of expression of MUPs (rather than the diversity of MUP isoforms) would be the trait under sexual selection. Further investigation is required to determine to what extent the detected variation in the expression of MUPs is genetic as opposed to phenotypic.

Acknowledgments We are grateful to Timothy Hort for language correction, Jaroslav Pialek, Milos Macholan, and Pavel Munclinger for helpful discussions. The work was funded by the Grant Agency of the Czech Republic (206-04/0493), whereas grant MSMT VZ 0021620828 from the Ministry of Education, Youth and Sport of the Czech Republic formed a framework for this study.

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J Chem Ecol (2007) 33:871–887
DOI 10.1007/s10886-007-9261-x

Alkaloids in Bufonid Toads (*Melanophryniscus*): Temporal and Geographic Determinants for Two Argentinian Species

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Received: 14 July 2006 / Revised: 9 October 2006 / Accepted: 5 November 2006 /
Published online: 28 February 2007
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Abstract Bufonid toads of the genus *Melanophryniscus* represent one of several lineages of anurans with the ability to sequester alkaloids from dietary arthropods for chemical defense. The alkaloid profile for *Melanophryniscus stelzneri* from a location in the province of Córdoba, Argentina, changed significantly over a 10-year period, probably indicating changes in availability of alkaloid-containing arthropods. A total of 29 alkaloids were identified in two collections of this population. Eight alkaloids were identified in *M. stelzneri* from another location in the province of Córdoba. The alkaloid profiles of *Melanophryniscus rubriventris* collected from four locations in the provinces of Salta and Jujuy, Argentina, contained 44 compounds and differed considerably between locations. Furthermore, alkaloid profiles of *M. stelzneri* and *M. rubriventris* strongly differed, probably reflecting differences in the ecosystem and hence in availability of alkaloid-containing arthropods.

Keywords Alkaloids · Ants · Arthropods · Bufonid toads · Mites

Introduction

Amphibians produce bioactive substances and in some cases sequester bioactive substances from dietary sources (Daly et al., 1987; Daly, 1995). Such substances appear to be stored in

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so-called granular or poison glands of the skin and are used as chemical defenses against predators and/or microbial infection. Substances produced by amphibians include a variety of antibiotic peptides (Bevins and Zasloff, 1990), distasteful biogenic amines and congeners (Roseghini et al., 1976, 1986), toxic steroidal bufadienolides (Flier et al., 1980; Daly et al., 2004), toxic samandarine alkaloids (Schöpf, 1961; Mebs and Pogoda, 2005), and the indolic pseudophrynamine alkaloids (Daly et al., 1990; Smith et al., 2002). The role of symbiotic microorganisms in biosynthesis of tetrodotoxins in amphibians remains unresolved. Evidence both for a role in symbiotic organisms (Daly et al., 1997b) and in production by an amphibian (Cardall et al., 2004) have been presented. The remarkable sequestration of a wide structural range of lipophilic alkaloids into skin glands of so-called poison frogs/toads of the neotropical dendrobatid genera *Dendrobates*, *Epipedobates*, *Minyobates*, and *Phyllobates* (Daly et al., 1994a,b; Jones et al., 1999 and unpublished) the Madagascan mantellid genus *Mantella* (Daly et al., 1997a), and undoubtedly the South American bufonid genus *Melanophryniscus* (Garraffo et al., 1993) suggests that these taxa either have overexpressed a primitive alkaloid-transport system or have independently evolved such a system. Myobatrachid frogs of the Australian genus *Pseudophryne* apparently have a different transport system that can transport pumiliotoxins, but not simple decahydroquinoline and izidine alkaloids that would occur in their ant diet (Smith et al., 2002).

Analysis of sequestered arthropod alkaloids has led to discovery and characterization of over 800 alkaloids during 40 year of investigation of skin extracts of poison frogs/toads (Daly et al., 2005). Fifty of such frog skin alkaloids have been detected in extracts of ants (Jones et al., 1999; Daly et al., 2000; Saporito et al., 2004; Clark et al., 2005), beetles (Dumbacher et al., 2004), millipedes (Saporito et al., 2003), mites (Takada et al., 2005), and various other arthropods (Daly et al., 2002).

The occurrence of alkaloids in different populations and species of poison frogs/toads provides information as to the distribution and availability of the arthropods that provide alkaloids to them. Thus, if species of the bufonid genus *Melanophryniscus* at a particular site and/or collection time do not have alkaloids known or presumed to be from a certain group of arthropods, then that mite, ant, beetle, or millipede is either not present or not targeted by the toad, perhaps, in the latter case because of more readily available arthropod prey that do not contain alkaloids. This question has been recently investigated and discussed both from a temporal and spatial standpoint for seven populations of the poison frog *Dendrobates pumilio* on a small island in the Bocas archipelago of Panamá (Saporito et al., 2006). Early studies of the alkaloid profile of *D. pumilio* extracts from some of the same populations also had indicated that distance between sites and the nature of habitat greatly influenced the profile (Daly et al., 2002). Similarly, the number of shared alkaloids in populations of the Colombian/Ecuadorian poison frog *Dendrobates histrionicus* was inversely related to the distance between collection sites, with frogs from sites close to each other sharing a greater number of alkaloids (Myers and Daly, 1976).

We report in this study both temporal changes in alkaloid profiles for one population of the bufonid toad *Melanophryniscus stelzneri* and spatial differences in alkaloid profiles for four populations of the bufonid toad *Melanophryniscus rubriventris*. The results are discussed in terms of possible arthropod food sources of the 72 alkaloids identified in extracts of these two toad species. Additional trace alkaloids were detected, but could not be adequately characterized. Structures of most of the major and minor alkaloids detected are shown (Fig. 1). Preliminary evidence for the postulated structures (Daly et al., 2005) of one pumiliotoxin, a dehydro-5,8-disubstituted indolizidine, and seven izidine alkaloids are presented.

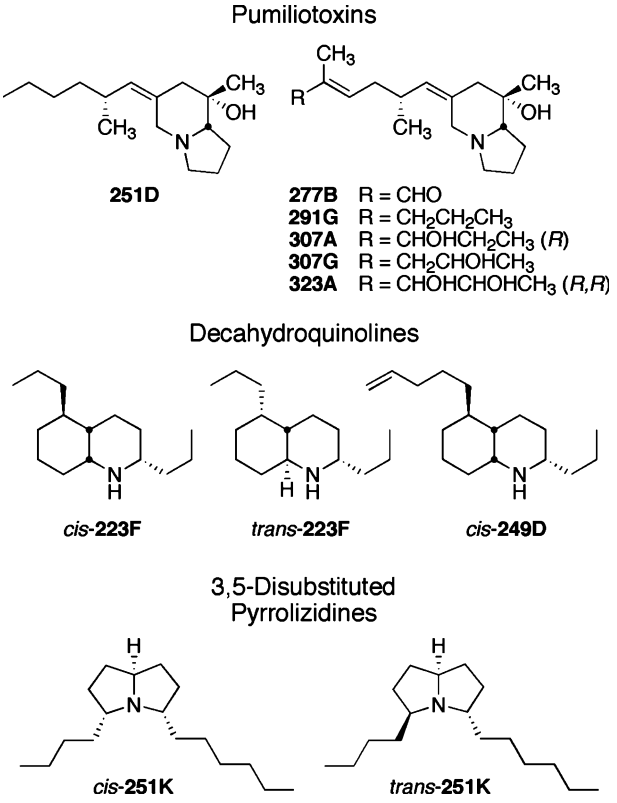


Fig. 1 Structures of major and minor alkaloids in collections of *Melanophryniscus stelzneri* and *Melanophryniscus rubriventris*. Postulated tentative structures of izidines **207S**, **207T**, and **221R** (Daly et al., 2005) are shown in Fig. 5. Structures of proposed tricyclics **203B**, **205H**, **221S**, **235I**, and **237O** and unclassified alkaloid **223T** are not known (see Daly et al., 2005)

Methods and Materials

Specimens of *Melanophryniscus stelzneri* were collected in May, 1989 (23 skins), and again in October, 1999 (eight skins), in fields with marshy streams and ponds during periods of heavy rain near the small town of Tanti, Córdoba, Argentina. Two males (combined skins) and two females (combined skins) were collected as amplexing pairs at the same site in November, 2001. *M. stelzneri* (31 skins) from another site in Córdoba were provided by Dr. Eduardo Gros.

Specimens from four populations of *M. rubriventris* were collected in 2002 in Argentina from forested sites at Cedral de Baritú (22° 29′ S, 64° 45′ W) (13 skins) and Canto del Monte (22° 22′ S, 64° 43′ W) (nine skins) in Salta and at Abra Colorada (23° 40′ S, 64° 53′ W) (11 skins) and Tiraxi (24° 01′ S, 65° 23′ W) (two skins) in Jujuy.

Skins were placed in methanol and extracts were subjected to acid-base partitioning as described (Garraffo et al., 1993). Compounds in the resultant alkaloid fraction were characterized by GC-MS and GC-FTIR spectral analysis (Garraffo et al., 1999). Arthropod stomach contents of *M. rubriventris* from the four sites were assessed as described (Bonanseal and Vaira, 2007).

Mass spectral data (EIMS, EI-MS/MS, CIMS (NH_3) and CI-MS/MS (NH_3)) were obtained with a Finnigan Thermoquest GCQ instrument, having a Restek RTX-5MS capillary column (30 m, 0.25 mm i.d.) programmed from 100° to 280° at 10° per min. GC-FTIR and EIMS spectra were obtained with a Hewlett–Packard model 5890 gas chromatograph, having

an HP-5 fused silica-bonded capillary column (30 m, 0.32 mm i.d.) programmed from 100 to 280° at a rate of 10° per min and interfaced with an HP model 5971 Mass Selective Detector and a Model 5965B IRD with a narrow band (4,000–750 cm^{-1}) detector. An HP ChemStation was used to generate EIMS and FTIR spectra.

Results and Discussion

Analysis of alkaloids in skin of the toad *Melanophryniscus stelzneri* collected in Tanti, Córdoba, Argentina, in 1989 was reported in 1993 (Garraffo et al., 1993) and has been updated in the present report with additional data (Table 1). Alkaloid profiles for two males and two females collected in 2001 were similar (Table 2). Profiles from another population of *M. stelzneri* from Las Alpacas, Córdoba were reported in 1993 (Garraffo et al., 1993). An updated profile is reported (Table 3).

The profile of alkaloids reported in 1993 (Garraffo et al., 1993) for *M. stelzneri montevidensis* from La Coronilla, Rocha, Uruguay, consists of pumiliotoxin **251D** and 3,5-disubstituted indolizidine 5*Z*,9*E*-**195B** as major alkaloids. Six trace alkaloids were also reported. This frog is now considered to be *Melanophryniscus montevidensis*, populations of which were recently reported to contain mainly pumiliotoxin **251D** (Mebs et al., 2005). However, pumiliotoxin **251D** in the La Coronilla population was present in only trace amounts (see below).

Table 1 Alkaloids in skin of *Melanophryniscus stelzneri* collected in Tanti, Córdoba, Argentina in 1989 and 1999

Melanophryniscus stelzneri Collected in Tanti, Córdoba	Alkaloids
23 Skins[a] (May, 1989)	
Pumiliotoxins	-/**251D**/-
Decahydroquinolines	*trans*-**223F**, *cis*-**249D**/*cis*-**223F**/*trans*-**249D**, *trans*-**249E**, *cis*-**275B**
3,5-Disubst. pyrrolizidines	*trans*-**251K**/*cis*-**223B**, *cis*-**251K**/*trans*-**223B**
3,5-Disubst. indolizidines	5*Z*,9*E*-**195B**/5*E*,9*Z*-**195B**, 5*Z*,9*Z*-**223AB**/5*Z*,9*Z*-**195B**, 5*E*,9*E*-**195B**
5,8-Disubst. indolizidines	**259B**/-/**207A**
Dehydro-5,8-disubst. indolizidines	-/**219G**/-
5,6,8-Trisubst. indolizidines	**235E**/-/-
Izidines	-/-/**207S**
Tricyclics	-/**193C**, **235I**/-
Spiropyrrolizidines	-/**236**/-
8 Skins (October, 1999)	
Pumiliotoxins	-/**251D**/-
3,5-Disubst. pyrrolizidines	-/*cis*-**251K**, *trans*-**251K**/-
5,8-Disubst. indolizidines	-/**259B**/**207A**
Dehydro-5,8-disubst. indolizidines	**219G**/-/-
5,6,8-Trisubst. indolizidines	**235E**/-/**207C**
Izidines	-/**207S**/**193I**
Tricyclics	-/-/**235I**

Major alkaloid > 50 μg per 100 mg skin; minor alkaloid 5–50 μg per 100 mg skin; trace alkaloid < 5 μg per 100 mg skin.

[a] Data updated from Garraffo et al., 1993.

Table 2 Comparison of skin alkaloids from male and female *Melanophryniscus stelzneri* collected near Tanti in 2001

Alkaloid	Males			Females		
	Major	Minor	Trace	Major	Minor	Trace
Pumiliotoxins	**251D**				**251D**	
3,5-Disubst. pyrrolizidines		*cis*-**251K**, *trans*-**251K**		*cis*-**251K**	*trans*-**251K**	
3,5-Disubst. indolizidines			**195B**			**195B**
5,8-Disubst. indolizidines			**207A**[a]			**193M**[b], **207A**[a]
Dehydro-5,8-disubst. indolizidines		**219G**				**219G**
5,6,8-Trisubst. indolizidines	**235E**		**223X**, **235E′**[c], **237S**, **251EE**	**235E**	**223X**	**207C**, **235E′**[c], **237S**, **251EE** (2)
Izidines		**207S**			**207S**	**193I**[b]
Tricyclics		**235I**	**193C**[b]		**235I**	
Unclass		**233K**[a], **267Y**	**223W**, **231J**[b], **247M**[b], **249P** (2)[b], **255F**[b], **265BB**		**223W**	**233K**[a], **247L**[b], **265BB**, **267Y**

M. stelzneri (two males/two females), Tanti, Córdoba, November, 2001. Major alkaloid > 50 μg per 100 mg skin; minor 5–50 μg per 100 mg skin; trace < 5 μg per 100 mg skin.

[a] Tentative identification.

[b] Not detected in the other sex.

[c] The prime in **235E′** indicates a trace isomer, in this case, after **235E** on GC analysis.

GC-MS chromatograms for the alkaloid fractions from the 1989 and 1999 collections of *M. stelzneri* are shown (Fig. 2). The toad and the collection site near Tanti are shown in Fig. 3. The site reportedly had undergone little obvious change from 1989 to 1999. Some major and minor alkaloids were present in both the 1989 and 1999 extracts, namely pumiliotoxin **251D**, both *cis*- and *trans*-isomers of the 3,5-disubstituted pyrrolizidine **251K**, the 5,8-disubstituted indolizidine **259B**, the dehydro-5,8-disubstituted indolizidine **219G**, and the 5,6,8-trisubstituted indolizidine **235E** (Table 1). The pumiliotoxins and the 5,8-

Table 3 Alkaloids in skin of *Melanophryniscus stelzneri* collected in Las Alpacas, Córdoba, Argentina

M. stelzneri (31 Skins)[a] (November, 1987)	Alkaloids
Pumiliotoxins	-/-/**251D**
Decahydroquinolines	*cis*-**223F**, *trans*-**223F**/*cis*-**249D**/*trans*-**249D**, *trans*-**249E**, *cis*-**275B**
3,5-Disubst. Pyrrolizidines	-/-/*trans*-**223B**, *cis*-**223H**, *trans*-**251K**
3,5-Disubst. Indolizidines	5*Z*,9*E*-**223AB**/5*E*,9*Z*-**195B**, 5*Z*,9*E*-**195B**, 5*E*,9*Z*-**223AB**/5*Z*,9*Z*-**195B**, 5*E*,9*E*-**195B**
5,8-Disubst. Indolizidines	-/**207A**/**259B**
5,6,8-Trisubst. Indolizidines	-/-/**235E**
Tricyclics	-/-/**193C**, **235I**

Major alkaloid > 50 μg per 100 mg skin; minor alkaloid 5–50 μg per 100 mg skin; trace alkaloid < 5 μg per 100 mg skin.

[a] Data updated from Garraffo et al., 1993.

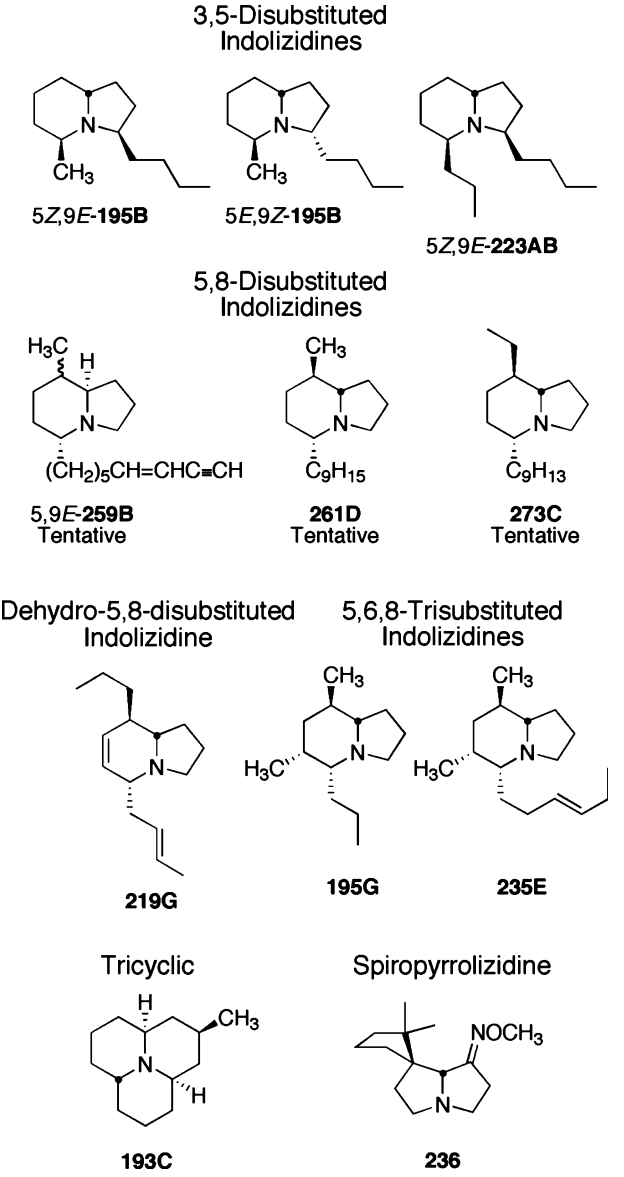


Fig. 2 GC-MS traces for alkaloids from the 1989 and 1999 collections of *Melanophryniscus stelzneri*. Alkaloids responsible for the major/minor peaks are indicated

disubstituted- and 5,6,8-trisubstituted-indolizidines are likely of mite origin (Takada et al., 2005; Saporito et al., 2006), while the 3,5-disubstituted pyrrolizidines are likely of ant origin (Jones et al., 1999). However, all of the decahydroquinolines, likely of ant origin (Jones et al., 1999), present in abundance in the 1989 extract, were completely absent from the 1999 extract (Table 1). The 3,5-disubstituted indolizidines, a class likely of ant origin (Jones et al., 1999), present in the 1989 extract, were also absent from the 1999 extract. Finally, the beetle alkaloid precoccinelline (**193C**), and the millipede spiropyrrolizidine alkaloid **236** were absent in the 1999 extract. The most likely explanation is that the ants, beetles, and millipedes had come to represent a less available source of alkaloids during the 10-year period at this site, while perhaps mites had become a more dominant source of



Fig. 3 A *Melanophryniscus stelzneri* toad from Tanti and the collection site

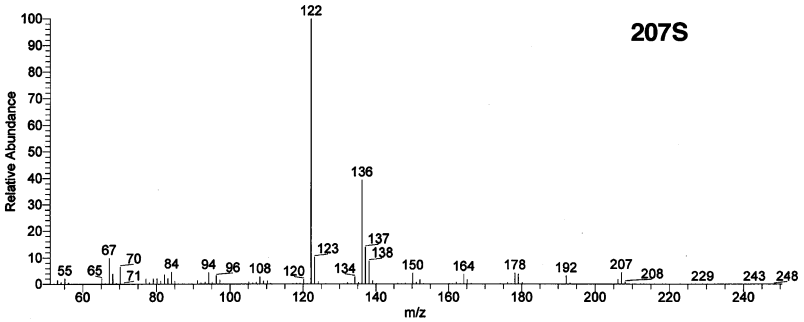
alkaloids for *M. stelzneri*. The arthropod stomach contents of *M. stelzneri* from "El Trapiche", San Luis, Argentina, have been reported (Filipello and Crespo, 1994). The three most common categories of prey items were ants, mites, and Collembola.

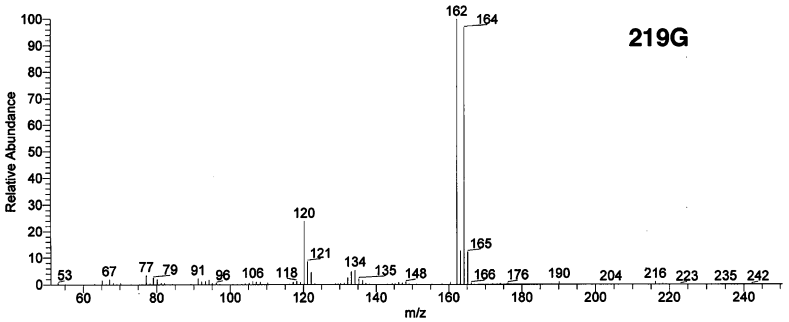
The proposed structure for the dehydro-5,8-disubstituted indolizidine **219G** (Daly et al., 2005) present as a minor alkaloid in the 1989 extract and as a major one in the 1999 extract, is based on chemical and spectral properties. The structure is shown (Fig. 1), as are the EIMS and FTIR spectra (Fig. 4). The FTIR band at 963 cm^{-1} indicates a *trans*-double bond. About 30 skin alkaloids are now assigned to the dehydro-5,8-disubstituted indolizidine class (Daly et al., 2005).

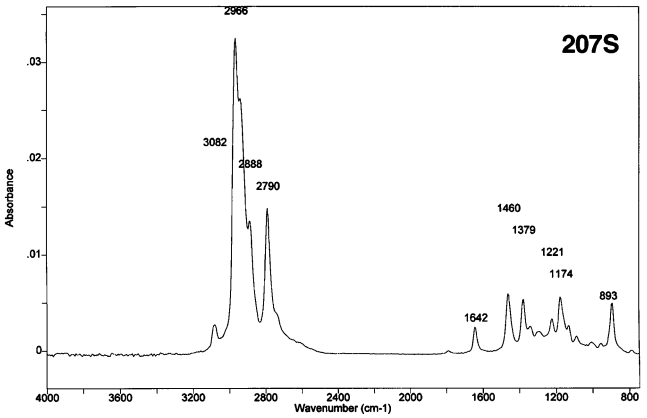
Two alkaloids (**193I** and **207S**) in the 1999 extract have been proposed to be "izidines" (Daly et al., 2005), based on GC-MS spectral properties, and for **207S** also on GC-FTIR spectral properties. The spectra of **207S** are shown (Fig. 4). The proposed structures (Fig. 5) are tentative, and isolation and NMR spectral analysis will be required to establish absolute structures.

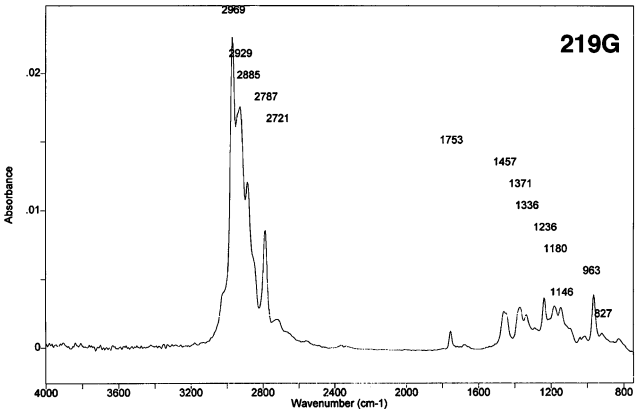
The males/females from two amplexing pairs collected in 2001 from the site near Tanti had similar profiles (Table 2). Twenty-eight alkaloids, including isomers, were identified. All major/minor alkaloids except the unclassified alkaloids **233K** and **267Y** had been detected in the 1989 and/or 1999 collections. Nine of the 14 trace alkaloids (six in males, three in females) were detected in only one sex. Seven of the trace alkaloids were detected for the first time in four decades of analysis of anuran skin alkaloids. Tentative structures for the two new indolizidines **193M** and **251EE** are depicted (Fig. 5). The properties of the seven trace alkaloids (in the format of the supporting information of Daly et al., 2005) that were previously unreported are as follows: **193M**. 5,8-I. '$C_{13}H_{23}N$'. Rt 7.26. MS: 193(1), 178(3), 151(21), 138(100), 96(30). 0D. Bufonid; **247L**. Unclass. '$C_{17}H_{29}N$'. Rt 12.18. MS: 247(100), 218(6), 204(48), 190(17), 176(87), 162(13), 134(11), 120(33), 94(14). 0D. Bufonid; **247M**. Unclass. '$C_{17}H_{29}N$'. Rt 11.65. MS: 247(14), 232(39), 206(100), 180(8), 84 (3). 0D. Bufonid; **251EE**. 5,6,8-I. '$C_{16}H_{29}NO$'. Rt 11.37. MS: 251(5), 168(100), 150(31), 110(5), 70(5). 1D. Bufonid; **255F**. Unclass. '$C_{15}H_{29}NO_2$'. Rt 11.47. MS: 255(3), 226(100), 196(8), 168(36), 166(81), 126(13), 86(5), 84(7). 1D. All major fragments have 1D. Bufonid; **265BB**. Unclass. $C_{16}H_{27}NO_2$. Rt 13.67. MS: 265(4), 250(2), 208(10), 180(76), 152(25), 140(15), 112(43), 98(36), 96(15), 86(100). 0D. Bufonid; **267Y**. Unclass. $C_{16}H_{29}NO_2$. Rt 12.40. MS: 267(21), 252(2), 210(100), 182(9), 138(10). 0D. Bufonid.

An updated alkaloid profile for a 1987 collection of *M. stelzneri* from Las Alpacas, Córdoba, Argentina (Garraffo et al., 1993) is shown (Table 3). The exact location of this


207S
Relative Abundance
m/z
122
136
137
138
123
67
70
71
55
65
84
94
96
108
120
134
150
164
178
192
207
208
229
243
248


219G
Relative Abundance
m/z
162
164
120
121
165
53
67
77
79
91
96
106
118
134
135
148
166
176
190
204
216
223
235
242


207S
Absorbance
Wavenumber (cm-1)
2966
3082
2888
2790
1460
1379
1221
1174
893
1642


219G
Absorbance
Wavenumber (cm-1)
2969
2929
2885
2787
2721
1753
1457
1371
1336
1236
1180
963
1146
827

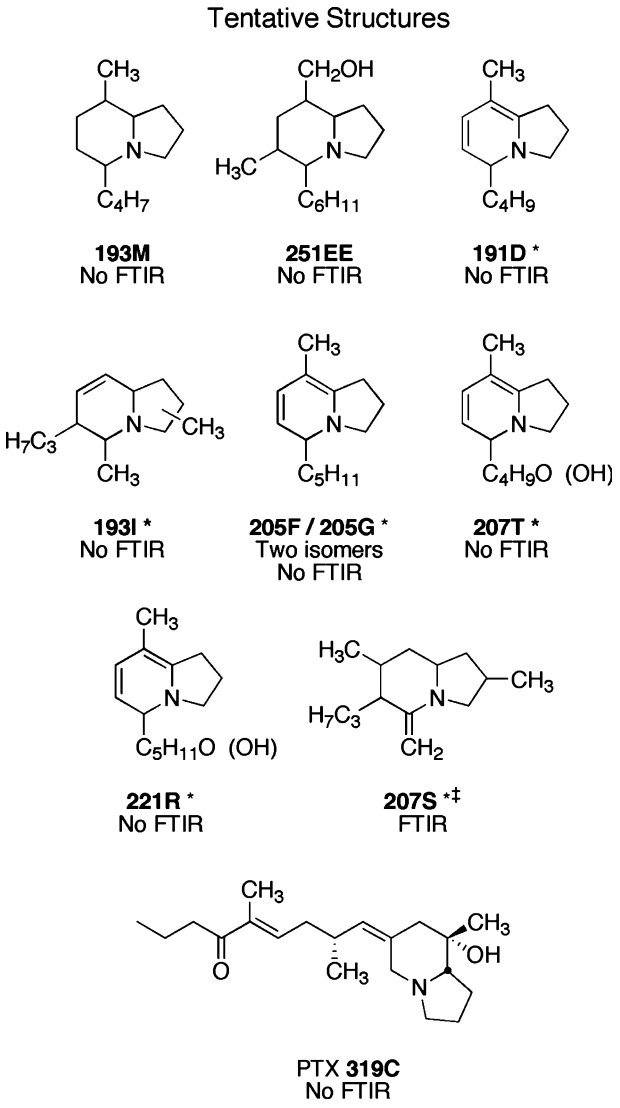


Fig. 5 Tentative structures for izidines and a pumiliotoxin in collections of *Melanophryniscus stelzneri* and *Melanophryniscus rubriventris*. Structures for those indicated by an apostrophe (*) were postulated (Daly et al., 2005), based on GC-MS and in some cases GC-FTIR data. ‡High resolution MS data now obtained

site is uncertain. Decahydroquinolines, pyrrolizidines, and indolizidines comprise 18 of the 21 alkaloids detected. All major alkaloids in this extract are likely of ant origin (cf., Jones et al., 1999).

The profile of alkaloids in a 1987 collection of *M. montevidensis* from La Coronilla, Rocha, Uruguay, was quite different. Pumiliotoxin **251D** and 3,5-disubstituted indolizidine 5*Z*,9*Z*-**195B** were the only major alkaloids (Garraffo et al., 1993). There were six others, all in trace amounts. These consist of three homopumiliotoxins **319A**, **319B**, and **321B**, the 5*E*,9*Z* isomer of indolizidine **195B**, and two 3,5-disubstituted pyrrolizidines, *cis*-**223H** and **237G**. Structures for the homopumiliotoxins have been proposed (Garraffo et al., 1993; Daly et al.,

◀**Fig. 4** The MS and FTIR spectrum of izidine **207S** and dehydro-5,8-indolizidine **219G**

Table 4 Alkaloids in skin of *Melanophryniscus rubriventris* from four locations in NW Argentina (Major/Minor/Trace)

Melanophryniscus rubriventris from Four Locations in NW Argentina	Alkaloids
M. rubriventris (11 skins), Abra Colorada, Jujuy, 2002	
Pumiliotoxins	**307G**, **323A**/**277B**/-
5,8-Disubst. indolizidines	-/**273C**/-
5,6,8-Trisubst. indolizidines	-/-/**195G**
Izidines	-/**207T**/**191D**, **205F**, **207T′**
Tricyclics	-/-/**221W**
M. rubriventris (13 skins), Cedral de Baritú, Salta, 2002	
Pumiliotoxins	**291G**/**307G**/**237A**, **289C**, **307A**, **309A**
5,6,8-Trisubst. indolizidines	-/-/**195G**
Izidines	-/**221R**/**205G**
Tricyclics	-/**205H**/**203B**, **207U**
M. rubriventris (9 skins), Canto del Monte, Salta, 2002	
Pumiliotoxins	**291G**, **307G**/**251D**/**237A**, **277B**, **277G**, **289C**, **307A**, **309A**
Deoxyhomopumiliotoxins	-/-/**207O**
3,5-Disubst. indolizidines	-/-/5*Z*,9*Z*-**223AB**
5,6,8-Trisubst. indolizidines	-/-/**195G**,**207C**, **221Q**
Izidines	-/-/**191E**
Tricyclics	-/**203B**, **205H**, **221S**/**205H′**
Unclass	-/**223T** (2)/**183C**, **195L**, **209M** (2)
M. rubriventris (2 skins), Tiraxi, Jujuy, 2002	
Pumiliotoxins	**251D**, **323A**/**277B**, **307G**/**277B**, **307G**/**275H**, **275H**, **219G**, **319F**
Allopumiliotoxins	-/-/**267A**
5,8-Disubst. indolizidines	-/**261D**/**261D′**
5,6,8-Trisubst. indolizidines	-/-/**195G**, **221Q**
Izidines	-/-/**209N**, **221R**
Tricyclics	**193C**/**237O** (2)/**193C′**
Unclass	-/-/**183C**, **267R**

2005). The indolizidines and pyrrolizidines are undoubtedly of ant origin (cf., Jones et al., 1999), while pumiliotoxin **251D** appears likely to be of mite origin (cf., Takada et al., 2005; Saporito et al., 2006) as are probably the structurally similar homopumiliotoxins.

Recently, alkaloids detected in whole body methanolic extracts of 81 *M. montevidensis* collected from six sites in Uruguay from October, 2003, to February, 2004, were reported (Mebs et al., 2005). La Coronilla was one of the six sites. Pumiliotoxin **251D** and indolizidine 5*Z*,9*E*-**195B** were major alkaloids (>50 μg/toad skin) in extracts from 39 skins of *M. montevidensis* collected in 1987 (see above) and supplied to us by Vittorio Erspamer (Garraffo et al., 1993). None of the whole body extracts from 20 individual toads collected in 2003–2004 had more than trace amounts (1 g/toad or less) of pumiliotoxin **251D** or of an unidentified indolizidine (perhaps **195B**, which was a major alkaloid in the 1987 collection). This seems to represent a remarkable temporal change in alkaloid content,

Fig. 6 The GC-MS traces for the alkaloids from the four populations of *Melanophryniscus. rubriventris*. Alkaloids responsible for most of the major/minor peaks are indicated. *A siloxane derivative formed on the GC column from pumiliotoxin **323A**

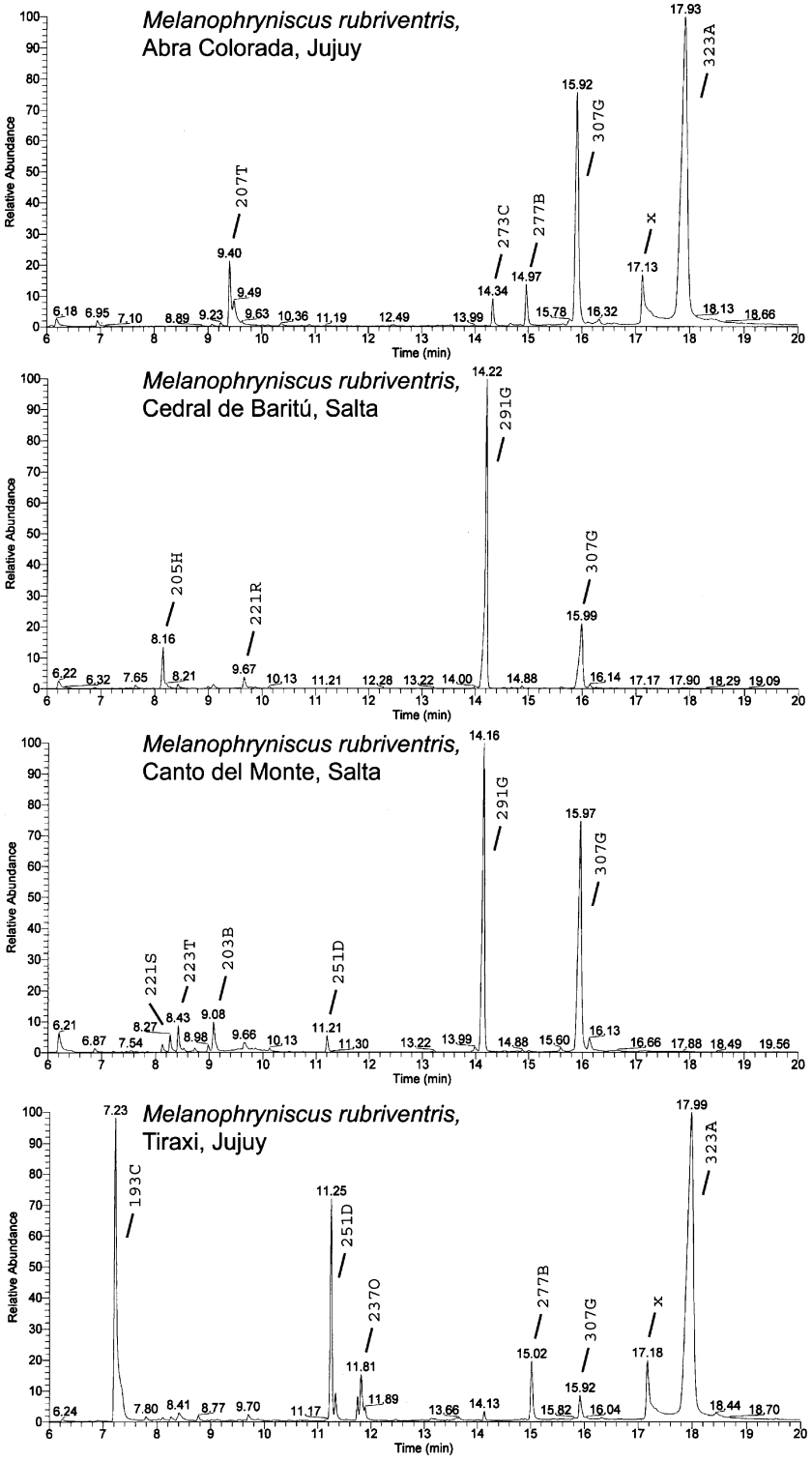

Melanophryniscus rubriventris,
Abra Colorada, Jujuy
Melanophryniscus rubriventris,
Cedral de Baritú, Salta
Melanophryniscus rubriventris,
Canto del Monte, Salta
Melanophryniscus rubriventris,
Tiraxi, Jujuy
Relative Abundance
Time (min)
207T
273C
277B
307G
323A
205H
221R
291G
221S
223T
203B
251D
193C
237O
x



Fig. 7 *Melanophryniscus rubriventris* toads from the four collection sites. **A**. Abra Colorada. **B**. Cedral de Baritú. **C**. Canto del Monte. **D**. Tiraxi

although it is uncertain whether the locations of the La Coronilla sites are exactly the same. Toads from two of the more southern sites had variable, but in some cases very high levels (>400 g/toad) of pumiliotoxin **251D** (Mebs et al., 2005).

The profile of alkaloids in the four different populations of *M. rubriventris* differed markedly (Table 4). GC-MS chromatograms are shown (Fig. 6) and toads from each site illustrate the variation in color and patterns (Fig. 7). The known distribution of the species in Argentina is confined to the upland portion of northwestern subtropical montane forests (Andean Yungas) and limited to six disjunct areas. Three populations are known in the north and central regions of Salta, and three are in the southeast of Jujuy. All are between 1,000 to 2,000 m elevation (Vaira, 2002). A map shows the four collection sites (Fig. 8). The collection site near Tanti, Córdoba, for *M. stelzneri* is also included in this map. The sites at which *M. rubriventris* are found, usually are well-structured cloud forests with richness in vegetation species and high vegetation density. Such steep, forested slopes are dominated by an evergreen-shaded forest, with a lush epiphytic growth mainly of bromeliads, ferns, mosses, and liverworts. Moderately steep portions of the forest allow temporary ponds that *M. rubriventris* use as breeding sites. The toads also are relatively common and easily seen in secondary forests, even on partly altered habitats, such as along montane trails or roads (Lavilla and Vaira, 1997). Toads choose small shallow ponds with rooted herbs for egg deposition. These little ponds are filled only during spring and summer rainstorms (Vaira, 2005). Further detailed studies on the botanical composition and arthropod occurrence at sites of the *Melanophryniscus* collections are needed. The spectrum of arthropods in the stomachs of toads from each of the collection sites are reported (Table 5). The spectra differed in terms of proportions of the two major prey items, ants, and mites.

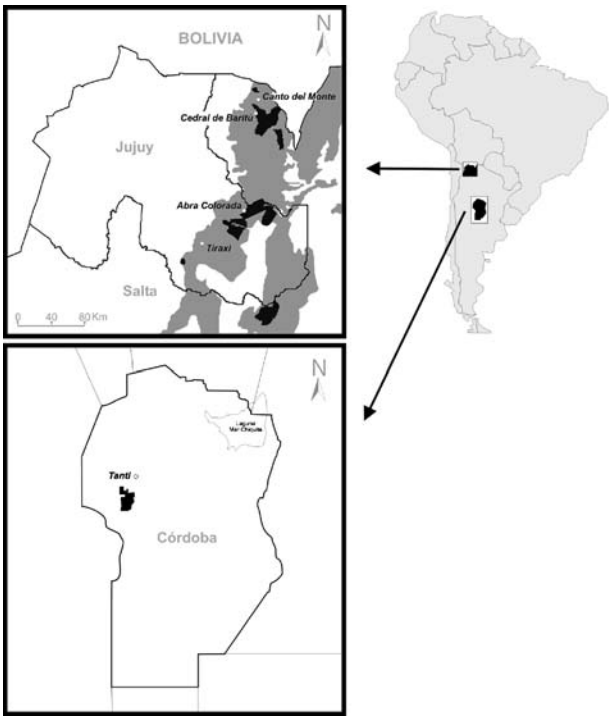


Fig. 8 Collection sites in Argentina for *Melanophryniscus stelzneri* and *Melanophryniscus rubriventris*. Protected areas in black. NW subtropical montane forests in black and in grey

Pumiliotoxins were present as major alkaloids at each site. Pumiliotoxin **307G** was a major alkaloid in extracts of toads from Abra Colorada, Cedral de Baritú, and Canto del Monte, and a minor one in extracts from Tiraxi. Other major pumiliotoxins were **323A** from Abra Colorada and Tiraxi, **291G** from Cedral de Baritú and Canto del Monte, and **251D** from Tiraxi. If mites are the source of the pumiliotoxins, different species might be used as dietary sources at the 4 sites. Only in the extract from Tiraxi, a trace of the allopumiliotoxin **267A** was detected, while a trace of the deoxyhomopumiliotoxin **207O** was detected only in the extract from Canto del Monte. One of the trace pumiliotoxins (**319C**) was detected for the first and, as yet, only time in these *M. rubriventris* extracts. This alkaloid was reported as unclassified with an unknown structure (Daly et al., 2005). Further analysis of the data indicates that it is a pumiliotoxin. A tentative structure is depicted (Fig. 5).

Decahydroquinolines, presumably of ant origin, were absent from the extracts of *M. rubriventris*, as were the ant-derived 3,5-disubstituted pyrrolizidines and 3,5-disubstituted indolizidines, with the sole exception being a trace amount of the 3,5-disubstituted indolizidine **223AB**, in the extract from Canto del Monte. In contrast, the 5,8-disubstituted indolizidines **261D** and **273C** occurred as minor alkaloids, each in one extract, while the 5,6,8-trisubstituted indolizidine **195G** occurred as a trace alkaloid in all four extracts. Two other 5,6,8-trisubstituted indolizidines (**207C** and **221Q**) also occurred in trace amounts in one or two extracts. Such indolizidines with branch points in the carbon skeleton are suspected to be of mite origin (Takada et al., 2005; Saporito et al., 2006). Several izidines (**191D**, **191E**, **205F**, **205G**, **207T**, and **221R**) occurred in minor or trace amounts usually in only one extract with the exception of **221R**, which occurred in two. Possible izidine structures have been proposed for some (Daly et al., 2005) and are shown (Fig. 5). All except **205F** and **205G** had not been detected previously and, thus, like pumiliotoxin **319C**, are as yet known only from *M. rubriventris*.

Table 5 Number of arthropods in stomach contents (expressed as percentage of each prey category by number in the total prey) from four populations of *Melanophryniscus rubriventris* in NW Argentina

Prey Categories	Abra Colorada (20)	Cedral de Baritú (26)	Canto del Monte (13)	Tiraxi (59)
Acari	35.6	8.3	39.6	20.0
Araneae	0.4	1.3	1.6	1.4
Coleoptera	8.7	6.5	4.5	4.7
Collembola	6.9	0.0	4.2	22.0
Diptera	0.7	0.0	0.3	0.3
Hemiptera	6.9	3.3	5.3	39.2
Hymenoptera (no Formicidae)	2.9	1.3	2.9	6.4
Formicidae	37.5	79.3	41.1	5.9
Plecoptera	0.4	0.0	0.0	0.0
Miriapoda	0.0	0.3	0.4	0.0

The numbers in parentheses indicate the number of toads examined.

Tricyclics were present as major or minor alkaloids in all extracts of *M. rubriventris* except the one from Abra Colorada, where tricyclic **221W** was present as a trace alkaloid. Presumably, the tricyclics, in particular precoccinelline (**193C**), a major alkaloid in the Tiraxi extract, are of beetle origin (Ayer and Browne, 1977). However, precoccinelline and another tricyclic alkaloid were recently reported from an oribatid mite (Takada et al., 2005). Except for **193C**, structures for all proposed tricyclics from *Melanophryniscus* species are not known (Daly et al., 2005).

The results suggest that the nature and/or availability of dietary sources of alkaloids found in skin of *Melanophryniscus* toads is strongly dependent on the site of collection and can change markedly with time. Alkaloid profiles from other sites and species of *Melanophryniscus* have been determined (Garraffo et al., 1993; Mebs et al., 2005; and for *Melanophryniscus klappenbachi* and *Melanophryniscus cupreuscapularis*, unpublished data with J. Faivovich and P. Cacivio). Such profiles differ markedly from the alkaloid profiles in the two species of the present report.

Individual variability in alkaloid profiles within a population does occur in *Melanophryniscus* (Mebs et al., 2005) and also in dendrobatid (Saporito et al., 2006) and mantellid frogs (Clark et al., 2005). Remarkable variability in levels of pumiliotoxin **251D** has been reported for 81 individual *M. montevidensis* from Uruguay (Mebs et al., 2005). The occurrence in trace amounts or nondetection of six other pumiliotoxins was also reported. An undefined indolizidine x was detected in all extracts, but apparently only in trace amounts. Pumiliotoxin **251D** varied from undetectable in certain individuals and present in trace amounts in others (<1 g/toad) from the two northern populations to levels of over 200 g/toad in individuals of three of the four southern populations. Mean values for the southern populations ranged from 29±16 g/toad (only two individuals) and 44± 21 g/toad (17 individuals) to very high levels of 198±51 g/toad (11 individuals) and 307± 34 g/toad (29 individuals). Obviously, both site and individual variations were considerable for pumiliotoxin **251D** in that study on *Melanophryniscus*. Pumiliotoxins were not detected in small arthropods, mainly ants collected in "various areas of Uruguay". Mites are the probable dietary source of pumiliotoxins (Takada et al., 2005; Saporito et al., 2006). However, only a limited number of mites were analyzed. Further studies on the factors responsible for temporal, individual, and site variation are needed.

The arthropod stomach contents of *M. rubriventris* are summarized (Table 5). Arthropod stomach contents were essentially similar in number of prey categories, but with a noticeable interpopulation diet variation with respect to the proportions of each prey item consumed. The high proportion of ants and low proportion of mites in the diet of toads from Cedral de Baritú was striking. However, pumiliotoxins, thought to be of mite origin, were the dominant alkaloids (Table 4). Perhaps, no simple correlation exists between the number of prey items and the alkaloid content in toad skin because some of the minor prey items might have very high alkaloid content and major prey items might be devoid of alkaloids. Toads from Tiraxi had a more generalized diet than the other populations. Ants represented a minor component of diet. Instead Acari (mites), hemipterans, and collembolans were the main prey categories. Pumiliotoxins thought to be of mite origin were the dominant alkaloids. Such differences in decreased consumption of ants may reflect changes in the composition of the arthropod assemblage or species availability.

Consumption of a different array of prey items could also be a consequence of changes in foraging habits in the individuals from the Tiraxi population. Prey-searching behavior is unknown for *M. rubriventris*, but adults are mainly diurnal and mobile with most of their reproductive activity taking place during daylight over the entire breeding season (Vaira, 2005). However, one of us repeatedly registered events of breeding activity at night in the population of Tiraxi (M.V. personal observation). Differences in diet for this population might reflect foraging adaptations to nocturnal habits, and consequently different prey communities, rather than prey selectivity or differential availability of arthropod species.

Further studies on the dietary origin of the arthropod alkaloids sequestered into anuran skin are needed. The factors that govern availability of such alkaloid-containing arthropod prey may help to understand better the ecology of *Melanophryniscus* and other anurans that depend either in part or exclusively on a diet of alkaloid-containing arthropods for protection against predators. Dendrobatid and mantellid poison frogs apparently have no other noxious substances beyond the alkaloids sequestered from their diet. However, toads of the genus *Melanophryniscus*, like other bufonids (Daly et al., 2004), have high levels of toxic bufadienolides or bufadienolide-like compounds (Flier et al., 1980), which would protect populations even where alkaloid-containing prey are not available. Myobatrachid frogs of the genus *Pseudophryne* produce their own pseudophrynamine alkaloids, in addition to sequestering pumiliotoxins from dietary arthropods (Smith et al., 2002). The evolutionary forces that led in four different anuran lineages to sequestration and use of dietary alkaloids in chemical defense remains intriguing and enigmatic.

Acknowledgments J.M.W. was supported by the NIH Undergraduate Scholarship Program. Fieldwork on *M. rubriventris* was done as part of a project supported by a grant from CONICET to M.V. Permits for sample collection of the species were provided by Delegación Técnica de Parques Nacionales, Regional Noroeste, Argentina. The research at NIH was supported by the intramural program of NIDDK.

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J Chem Ecol (2007) 33:889–907
DOI 10.1007/s10886-007-9275-4

Using Generic Pheromone Lures to Expedite Identification of Aggregation Pheromones for the Cerambycid Beetles *Xylotrechus nauticus*, *Phymatodes lecontei*, and *Neoclytus modestus modestus*

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Received: 9 January 2007 / Accepted: 23 February 2007 /
Published online: 29 March 2007
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Abstract Males of several species of longhorned beetles in the subfamily Cerambycinae produce sex or aggregation pheromones consisting of 2,3-hexanediols and/or hydroxyhexanones. We tested the hypothesis that this diol/hydroxyketone pheromone motif is highly conserved within the subfamily, and the resulting prediction that multiple cerambycine species will be attracted to compounds of this type. We also tested the concept that live traps baited with generic blends of these compounds could be used as a source of live insects from which pheromones could be collected and identified. Traps placed in a mature oak woodland and baited with generic blends of racemic 2-hydroxyhexan-3-one and 3-hydroxyhexan-2-one captured adults of both sexes of three cerambycine species: *Xylotrechus nauticus* (Mannerheim), *Phymatodes lecontei* Linsley, and *Phymatodes decussatus decussatus* (LeConte). Odors collected from male *X. nauticus* contained a 9:1 ratio of two male-specific compounds, (*R*)- and (*S*)-3-hydroxyhexan-2-one. Field trials with synthetic compounds determined that traps baited with (*R*)-3-hydroxyhexan-2-one (94% ee), alone or in blends with other isomers, attracted similar numbers of *X. nauticus* of both sexes, whereas (*S*)-3-hydroxyhexan-2-one (94% ee) attracted significantly fewer beetles. *Phymatodes lecontei* and *P. d. decussatus* also were caught in traps baited with hydroxyhexanones, as well as a few specimens of two other cerambycine species, *Neoclytus modestus modestus* Fall (both sexes) and *Brothylus gemmulatus* LeConte (only females). Male *N. m. modestus* produced (*R*)-3-hydroxyhexan-2-one, which was not present

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in extracts from females. *Neoclytus m. modestus* of both sexes also responded to lures that included (*R*)-3-hydroxyhexan-2-one as one of the components. The only male-specific compound found in extracts from *P. lecontei* was (*R*)-2-methylbutan-1-ol, and adults of both sexes were attracted to racemic 2-methylbutan-1-ol in field bioassays. Surprisingly, *P. lecontei* of both sexes also were attracted to (*R*)- and (*S*)-3-hydroxyhexan-2-ones, although neither compound was detected in extracts from this species. Males of all five beetle species had gland pores on their prothoraces that were similar in structure to those that have been associated with volatile pheromone production in other cerambycine species. The attraction of multiple cerambycine species of two tribes to (*R*)-3-hydroxyhexan-2-one in this study, and in earlier studies with other cerambycine species, suggests that this compound is a widespread aggregation pheromone component in this large and diverse subfamily. Overall, the attraction of multiple species from different cerambycine tribes to this compound at a single field site supports the hypothesis that the hydroxyketone pheromone structural motif is highly conserved within this subfamily.

Keywords Aggregation pheromone · Cerambycidae · (*R*)-3-Hydroxyhexan-2-one · Insect communities · Sex pheromone · Woodborer

Introduction

Males of several longhorned beetle species in the subfamily Cerambycinae produce volatile sex or aggregation pheromones (Lacey et al. 2007a). These pheromones are comprised of one to three compounds that share a similar structural motif consisting of molecules that are six, eight, or 10 carbons in length, with hydroxyl or carbonyl groups at C_2 and C_3 (reviewed by Lacey et al. 2004, 2007a). The pheromones are produced by sex-specific glands in the prothorax of males that discharge through pores situated within depressions in the cuticle (Ray et al. 2006; Lacey et al. 2007b). A morphological survey of 65 cerambycine species revealed that 49 species in 14 tribes had analogous male-specific prothoracic pores, suggesting that volatile pheromones may play an important role in reproduction or other behaviors for many species in this subfamily (Ray et al. 2006). The Cerambycinae is represented by at least 1,100 species in North America alone (excluding Mexico; Yanega 1996). Given this taxonomic diversity, and the many cerambycine species that have male-specific gland pores, it is likely that multiple species that produce volatile pheromones will be present in any given geographic area.

In this paper, we report the results of studies that tested the hypothesis that the diol/hydroxyketone pheromone motif is highly conserved within the Cerambycinae, and the resulting prediction that multiple cerambycine species will be attracted to compounds of this type. We chose six-carbon compounds for this first test of the hypothesis, rather than eight- or ten-carbon homologs, because all 10 North American cerambycine species that we have worked with previously were found to produce C_6 pheromones (Lacey et al. 2004, 2007a; Ray et al. 2006; unpublished data). Thus, 2,3-hexanediols, 2-hydroxyhexan-3-ones, and 3-hydroxyhexan-2-ones were synthesized and deployed as trap lures in field bioassays. Beetles that were attracted to the test compounds were trapped live, and the volatiles that they released were collected and analyzed to identify the actual pheromone compounds produced by each species. We report here the species that responded to the synthetic hexanediol and hydroxyhexanone lures in field bioassays conducted in one area of southern California. We further report the identification of pheromone components for three of these cerambycine species, *Xylotrechus nauticus* (Mannerheim), *Phymatodes lecontei* Linsley,

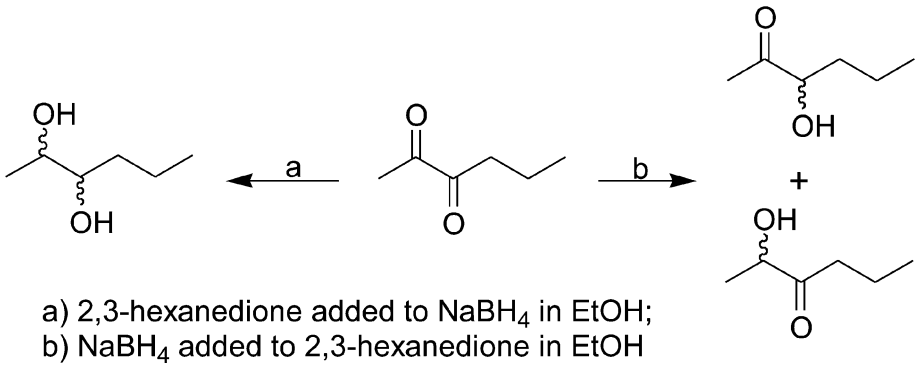
and *Neoclytus modestus modestus* Fall. We also characterize the morphology of the likely gland pores for each cerambycine species that was captured.

Methods and Materials

Synthetic Pheromones

A mixture of 2,3-hexanediols (henceforth generic hexanediols) was synthesized as follows (Scheme 1a). A solution of 2,3-hexanedione (114.15 g, 1.0 mol; Aldrich Chemical Co., Milwaukee, WI, USA; >90% purity) in ethanol (EtOH, 200 ml) was added over 3 hr to a stirred mixture of $NaBH_4$ (26.5 g, 0.70 mol) in EtOH (300 ml) at 0°C under argon. The reaction was warmed to room temperature and stirred overnight. The resulting mixture was concentrated, then poured into a stirred mixture of brine (500 ml) and 6 M HCl (200 ml), and extracted with ethyl acetate (5×200 ml). The combined organic phases were washed with saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. The residue was purified by Kugelrohr distillation (oven temperature 81–90°C, 3.0 mm Hg) affording 90.42 g (77% yield) of a mixture of racemic (2*R**,3*R**)-hexanediol and (2*R**,3*S**)-hexanediol (36:64, respectively: GC analysis on DB-5 column, 30 m×0.32 mm i.d., 0.25 μm film; J&W Scientific, Folsom, CA, USA; temperature program 40°C/1 min, 10°/min to 250°, helium carrier gas, head pressure 105 kPa, injector and detector temperatures, 250°C). The mass spectra and retention times matched those of standards (Lacey et al. 2004; GC-MS analysis, HP 5973 mass selective detector coupled to an HP 6890 GC [Hewlett-Packard, Avondale, PA, USA] fitted with an HP5-MS column, 30 m×0.25 mm i.d., 0.25 μm film, temperature program 40°C/1 min, 10°/min to 250°, helium carrier gas, 36 cm/sec linear flow, injector and transfer line temperatures, 250°C).

A blend of 2,3-hydroxyhexanones for bioassay trap lures (henceforth generic hydroxyhexanones), consisting of a mixture of racemic 2-hydroxyhexan-3-one and 3-hydroxyhexan-2-one, was synthesized as shown in Scheme 1b. A suspension of $NaBH_4$ (3.73 g, 99 mmol) in EtOH (80 ml) was added dropwise over 2 hr to a stirred solution of 2,3-hexanedione (30.00 g, 263 mmol; Aldrich Chem. Co.) in EtOH (60 ml) at 0°C. The resulting mixture was stirred for 8 hr at 0°C, and then allowed to warm to room temperature and stirred overnight. Water (150 ml) was added and the reaction was extracted with pentane:diethyl ether (Et_2O) (1:1–5×100 ml). The combined organic extracts were washed



Scheme 1 Synthesis of generic blends of 2,3-hexanediols and 2,3-hydroxyhexanones

with brine, dried over Na_2SO_4, and the solvent was removed by fractional distillation through a Vigreux column (fractional distillation was essential because concentration by rotary evaporation resulted in loss of the product). The residue was purified by vacuum flash chromatography (pentane/Et_2O 95:5 until all unreacted 2,3-hexanedione had been removed, then pentane/Et_2O 4:1). The solvent was removed by distillation through a Vigreux column, and the product was Kugelrohr distilled (oven temperature 40–45°C, 2.1 mm Hg) affording 13.81 g (45.2% yield) of a mixture of racemic 2-hydroxyhexan-3-one and 3-hydroxyhexan-2-one (59:41, respectively). Retention times and mass spectra of the two isomers matched those of authentic standards (Lacey et al. 2007a). The positional isomers were not separated on a DB-5 GC column, but both the positional isomers and the enantiomers were resolved on a chiral phase Cyclodex B column (30 m×0.25 mm i.d.× 0.25 μm film, J&W Scientific; temperature program 50°C/1 min, 5°/min to 250°, helium carrier gas, head pressure 150 kPa, injector temperature 100°C and detector temperatures 250°C). The four hydroxyhexanones eluted in the order (2*R*) (10.49 min), (3*R*) (10.56 min), (2*S*) (10.67 min), and (3*S*) (11.10 min). It was crucial to use an injector temperature of 100° C to prevent thermal isomerization of the hydroxyhexanones (Leal et al. 1995).

Racemic 3-hydroxyhexan-2-one and enantiomerically enriched (*R*)-3-hydroxyhexan-2-one and (*S*)-3-hydroxyhexan-2-one (both 94% ee) were synthesized as previously described (Lacey et al. 2007a). Racemic 2-methylbutan-1-ol (98%) was obtained from Alfa Aesar (Heysham, Lancashire, UK) and (*S*)-2-methylbutan-1-ol (99%) was obtained from Aldrich Chemical Co. The enantiomers of 2-methyl-1-butanol were resolved on the Cyclodex B column with a temperature program of 40°C/10 min, 5°/min to 200°C, with other operating conditions as described above. Under these conditions, the (*R*)-enantiomer eluted at 13.64 min, whereas the (*S*)-enantiomer eluted at 13.82 min.

Screening Bioassays to Target Species for Pheromone Identification

We targeted species for pheromone identification by baiting traps for collecting live insects with generic hexanediols, generic hydroxyhexanones, or a 1:1 blend of the two. The mixtures were diluted with absolute EtOH (Gold Shield Co., Hayward, CA, USA) (100 mg/ml for the single compounds, 100 mg/ml each for the blend), and loaded into lures (1 ml/lure) consisting of 0.5 dram glass vials with open-top screw caps closed with PTFE-faced silicone septa (Cat. no. 03-393F, Fisher Scientific, Fairlawn, NJ, USA). The septum was pierced with a 2-cm piece of glass tubing (2 mm i.d.), and a pipe cleaner wick (cotton and steel wire; 40 mm long) was inserted through the tubing. The basal 0.5 cm of the wick was bent at a 90° angle and was in contact with the bottom of the vial, and the other end protruded ~1 mm beyond the top of the glass tube. A preliminary experiment demonstrated that ethanol was an efficient carrier for the hexanediols and hydroxyhexanones, and that lures would release 1 ml of ethanol solution over the course of 2 d outdoors (conducted 23–25 May 2006, in shade, daily minimum air temperatures 13–15°C, maximum temperatures 26–31°C). Lures were hung with wire in cross-vane flight-intercept panel traps (black, 80× 30 cm, Intercept™, model PT, Advanced Pheromone Technologies, Marylhurst, OR, USA). To capture beetles alive, traps were modified by replacing the supplied collection basin with a 1.9-l plastic funnel with the spout shortened to give a 35-mm-diameter hole. The threaded lid ring from a wide mouth 960 ml canning jar (Alltrista Consumer Products Co., Muncie, IN, USA) was placed over the spout and glued to the bowl of the funnel so that the jar could be attached with the spout inside the jar. The upper halves of jar interiors, interior surfaces of funnels, and surfaces of trap panels were treated with Rain-X® (Houston, TX, USA) glass treatment to render them slippery (Sweeney et al. 2006). Trap hangers were

constructed of 1.27 cm i.d. PVC pipe (D1785, Schedule 40, Charlotte Pipe and Foundry Co., Charlotte, NC, USA), with a 1.5-m-long upright connected with a T-fitting to a 20-cm long arm having a loop of wire at the end to which the trap was attached. The upright was mounted on a 1.5-m section of 1.27-cm-diam steel reinforcing bar that was driven part way into the ground.

The three treatments (generic hexanediols, generic hydroxyhexanones, 1:1 blend of generic hexanediols and hydroxyhexanones) were randomly assigned to traps. Because the purpose of the experiment was to trap live beetles for pheromone collection, and not to identify specific attractants, controls were not used. A single set of traps was set up at the Santa Rosa Plateau Reserve (Riverside Co., CA, USA) in late afternoons of 4 d between 24 May and 2 June 2006, skies clear, maximum air temperatures 25–31°C. Traps were positioned 15 m apart in a southwesterly transect through a grassland area wooded with mature *Quercus agrifolia* Née (position of trap # 1: 33°31′58.12″N, 117°15′49.94″W). There were many dead trees and downed limbs of various ages at the site. Traps were checked daily for beetles, and captured beetles were taken to the laboratory and separated by species and sex into cylindrical aluminum screen cages (6-cm diam, 10 cm tall, with plastic Petri dishes at top and bottom). Beetles were provided a cotton roll saturated with 10% sucrose solution for water and nutrition. Treatments were rotated one position on each collection date.

Identification of Pheromone Components

Adult beetles of each sex were placed individually into two-piece glass vacuum traps (~0.3 l, ground glass fittings; UIUC School of Chemical Sciences glass shop) lined with aluminum screen as perches. A section of glass tubing containing 100 mg of 80/100 mesh SuperQ® (Alltech Associates, Deerfield, IL, USA) was attached to the outlet of each vacuum trap with a 2-cm long section of Teflon® tubing, and charcoal-purified air (6-14 mesh, Fisher Scientific, Pittsburgh, PA, USA) was pulled through the apparatus by vacuum at a rate of 1 l/min. The chambers were placed on the laboratory windowsill and volatiles were collected from adult beetles from 18:00 to 0:00 hr. The SuperQ® collectors then were eluted with 0.5 ml dichloromethane (CH_2Cl_2), and extracts were analyzed by gas chromatography-mass spectroscopy (GC-MS) on the HP5-MS column by using identical conditions as described earlier, except that injections were made in splitless mode and the injector temperature was 100°C. The absolute configurations of the insect-produced chiral compounds (see "Results") were determined by analysis of extracts on the Cyclodex B column, as described above and confirmed by co-injection of an aliquot of the insect extract spiked with the appropriate standards.

To quantify the amount of pheromone produced by *X. nauticus*, male beetles collected in field bioassays were placed individually in small aeration chambers constructed from 237-ml Ball® canning jars (Alltrista Consumer Products Co.) with two holes punched in the lid to accommodate 0.64 cm Swagelok® unions for attaching inlet and outlet lines. The inlet was fitted with a 15×1.5 cm copper tube filled with activated charcoal granules (6-14 mesh, Fisher Scientific, Pittsburgh, PA, USA), and the outlet was fitted with a trap consisting of a 4.5 cm×3 mm i.d. glass tube with a 5-mm plug of activated charcoal (50-200 mesh, Fisher Scientific, Pittsburgh, PA, USA) held in place by two glass wool plugs. Each chamber was equipped with a 1/2-dram vial filled with water, with the neck of the vial plugged with a cotton swab. Air was pulled through each chamber by vacuum at ~500 ml/min. Aerations were continued for 1–6 d (some beetles died during aerations), in a brightly lit environmental chamber at 25°C and ~50% RH, 14L:10D. Traps were eluted with 0.4 ml of CH_2Cl_2 into tared vials, and the vials were then reweighed to calculate the total weight.

Total volume of each extract was then determined by dividing the mass by the density of the solvent. One-microliter aliquots of each extract were analyzed by GC (as described above), and the total amount of pheromone in each extract was calculated by comparison with an external standard curve (5–500 μg/ml, regression r^2=0.996). Of 13 extracts collected, eight contained detectable amounts of pheromone.

Field Bioassays of Synthetic Compounds, Targeting Particular Species

We conducted field bioassays of synthetic compounds on 5 d between 13 and 19 June 2006, skies clear, maximum air temperatures 24–36°C. Compounds were formulated in EtOH, and lures were loaded with 1 ml of the resulting solutions. There were eight treatments that were randomly assigned to traps, as follows:

1) blank control (empty vial);
2) EtOH control;
3) racemic 2-methylbutan-1-ol (50 mg in 1 ml EtOH); although male *P. lecontei* produced (*R*)-2-methylbutan-1-ol (see "Results"), we used racemic 2-methylbutan-1-ol in lures because the (*R*)-enantiomer was not commercially available, and there was insufficient time left in the field season to synthesize the (*R*)-enantiomer for testing;
4) (*R*)-3-hydroxyhexan-2-one (25 mg/ml);
5) (*S*)-3-hydroxyhexan-2-one (25 mg/ml);
6) 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one (25 mg/ml; based on approximate ratios produced by male *X. nauticus*; see "Results");
7) 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one plus 2,3-hexanedione (22.5:2.5:6.6 mg, respectively, in 1 ml EtOH); 2,3-hexanedione was considered an artifact during pheromone identification of another cerambycine species (Fettköther et al. 1995), but was a possible minor component produced by male *X. nauticus* (see "Results");
8) generic blend of hydroxyhexanone stereoisomers (100 mg/ml; to determine whether responses of adult beetles would be influenced by the presence of the "unnatural" isomers).

We used three sets of eight traps (1 trap per treatment), with traps spaced 15 m apart in linear transects. One set of traps was in the same location as had been used for the screening bioassays (trap #1 in same position), and the second and third sets were set up among oak trees 0.42 km to the north and 0.54 km to the southwest, respectively, of trap # 1 of the first set. Thus, there was one replicate of each treatment in each of three sets of traps on each of 5 d (N=15 replicates per treatment). Treatments were rotated one position down the trap line on each collection date. Traps, funnels, and jars were cleaned with Windex® glass and surface wipes (S. C. Johnson & Sons, Inc., Racine, WI, USA), then treated with Rain-X®. Captured beetles were collected after 24 hr and the number and sex responding to each treatment were recorded. Lures were replaced as needed (when vials were empty, often after 24 hr).

Differences between treatments in numbers of beetles captured per trap were tested separately for the two most abundant species, *X. nauticus* and *P. lecontei*, by ANOVA (PROC GLM, SAS Institute 2001), blocked by sample day. Thus, the ANOVA model for each species was: number of beetles captured=treatment × day. We tested differences between preplanned pairs of treatment means by using orthogonal contrasts (Sokal and Rohlf 1995). Thus, we compared the EtOH control with the blank control (to determine whether EtOH itself was attractive), compared each treatment that contained synthetic compounds with the controls (blank and EtOH controls pooled because they did not differ significantly; see below), and also compared treatments containing (*R*)-3-hydroxyhexan-2-one with each other,

to determine effects of possible minor components or stereoisomers. We also compared the 2,3-hexanedione plus the 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one to the 9:1 blend alone. Structural failure of a few traps was responsible for slight inconsistencies in degrees of freedom for some analyses. Differences between treatments in the sex ratio of responding *X. nauticus* and *P. lecontei* were tested with χ^2 goodness-of-fit tests.

Location of Pheromone Glands

To confirm that sex-specific pores were present in the prothoraces of males of the species that were captured during the study (see "Introduction"), we imaged the prothorax of one adult of each sex by scanning electron microscopy (SEM). Specimens were prepared for SEM as described by Ray et al. (2006), and imaged with a Phillips XL30 environmental scanning electron microscope equipped with a field-emission electron gun (FEI Company, Hillsboro, OR, USA) operated at 5.0 kV.

To further confirm that pores in the prothorax of male *X. nauticus* and *P. lecontei* were associated with pheromone glands, we sectioned prothoraces of both sexes of each species for light microscopy. One male and one female were chilled, and the entire prothorax was excised. The prothorax samples were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, and held at <10°C, then later shipped on ice to the University of Illinois where they were stored at <10°C. After a 10-min buffer rinse, the samples were postfixed in 1% osmium tetroxide in the same buffer for 90 min in the dark. After a second buffer rinse, the samples were dehydrated through an extended ascending ethanol series (3 hr each in 37, 67, and 95% ethanol), culminating at 10 hr in 100% EtOH. The samples were then infiltrated and embedded in Poly/Bed® 812 embedding resin plus 2% (wt/wt) DMP-30 per manufacturer instructions (Polysciences 2003), with the exception that infiltration times were extended to 3 hr for each change, and a 6-hr immersion in fresh resin without DMP-30 was added before the final step. Prothoraces in embedding resin plus DMP-30 were then positioned horizontally in soft plastic molds (#105; Ted Pella, Inc., Redding, CA, USA) and placed in a 60°C oven for 24 hr. One-micron-thick sections were cut using an Ultracut UCT ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 6-mm histology grade diamond knife (MicroStar Technologies, Huntsville, TX, USA), affixed to standard glass microscope slides (Fisher Scientific, Fairlawn, NJ), and stained with a 1% toluidine blue solution. Sections were examined using a Nikon Eclipse E600 microscope (Nikon USA, Melville, NY, USA) operated in bright field mode, equipped with a SPOT Insight™ color camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Voucher specimens of all cerambycid species caught have been submitted to the Entomology Research Museum, University of California, Riverside.

Results

Screening Bioassays to Target Species for Pheromone Identification

Traps baited with generic lures captured three cerambycine species during the four trials (Table 1): *X. nauticus* (*N*=18), *P. lecontei* (*N*=30), and *Phymatodes decussatus decussatus* (LeConte) (*N*=6). Traps baited with the generic hydroxyhexanone lures and the blend of hydroxyhexanones and hexanediols contained the majority of the beetles caught in these bioassays, with only three beetles of one species (*P. lecontei*) caught in traps baited with the

Table 1 Cerambycine adults attracted to generic blends of possible pheromone components (screening experiment, *N*=4 days) or enantiomerically enriched pheromone components (targeted experiment, *N*= 5 days)[a]

Expt.	Treatment	*Xylotrechus nauticus*		*Phymatodes lecontei*		*Phymatodes decussatus*		*Neoclytus m. modestus*		*Brothylus gemmulatus*	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Screening	Generic hydroxyhexanones	5	3	5	12	3	3				
Screening	Generic hexanediols				3						
Screening	Generic hydroxyhexanones + generic hexanediols	5	5	5	5						
Targeted	Blank control	1	1		1						
Targeted	Ethanol control	1	4	2	5						
Targeted	2-Methylbutan-1-ol	1	3	19	12	3					
Targeted	(*R*)-3-Hydroxyhexan-2-one	16	28	27	38		1	2	1	1	
Targeted	(*S*)-3-Hydroxyhexan-2-one	9	6	24	12*		1				
Targeted	9:1 Blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one	27	18	19	37*			2		3	
Targeted	9:1 Blend of (*R*)- and (*S*)-, + 2,3-hexanedione	24	21	13	19			4	3		
Targeted	Generic hydroxyhexanones	30	23	16	28			2	1	2	

[a] Asterisks indicate statistically significant departures from 1:1 sex ratios (χ^2, $P<0.05$).

generic hexanediols alone (Table 1). A few specimens of other types of insects were caught in traps in this and the subsequent targeted studies, including earwigs, cockroaches, and bark beetles. These nontarget species were captured in all treatments, including blank controls. There was no indication that they were responding to the hydroxyhexanones or hexanediols, with the possible exception of a trogositid beetle, *Temnochila chlorodia* (Mannerheim), a few of which were captured in the targeted study (see below).

Identification of Pheromone Components

GC-MS analyses of extracts of volatiles collected from male *X. nauticus* typically showed a single major peak in the total ion chromatogram, which was absent in analogous headspace extracts of females (Fig. 1a). The retention time and mass spectrum of the compound (base peak at *m*/*z* 55, other significant fragments at *m*/*z* 43 [78%], 73 [53%], and 45 [26%]) matched those of a synthetic standard of 3-hydroxyhexan-2-one. The enantiomeric composition of the insect-produced compound was determined to be ~90% (*R*) and 10% (*S*) by analysis on the Cyclodex-B column, with baseline resolution of the enantiomers. In one extract with a particularly large amount of male-produced compounds (Fig. 1a), we also detected 2,3-hexanedione (~25% of the major peak), the identity of which was confirmed by comparison of its retention time and mass spectrum with those of an authentic standard. Under laboratory conditions, males produced 92±104 μg (mean ± S.D., range 2–310 μg/day, *N*=8), of 3-hydroxyhexan-2-one per day.

Collection and analysis of headspace volatiles from both sexes of *N. m. modestus* caught in targeted field trials (see below) revealed that males produced pure (*R*)-3-hydroxyhexan-2-one as the only detectable component (identified as described above for *X. nauticus*). This compound was not found in aeration extracts of females.

Extracts of headspace volatiles from male *P. lecontei* contained a single peak that was absent in analogous extracts from females (Fig. 1b). The compound was tentatively identified as 2-methylbutan-1-ol by matching its mass spectrum with a database spectrum (NIH-NBS mass spectral database), and the identification was confirmed by comparisons with the spectrum and retention times of an authentic standard. The absolute configuration of the insect-produced compound was determined to be (*R*) by analysis on the Cyclodex-B column. There was no evidence of hydroxyhexanones or hexanediols in extracts of headspace volatiles collected from *P. lecontei*.

Field Bioassays of Synthetic Compounds Targeting Particular Species

Five species of cerambycines were caught in the targeted field bioassays (Table 1), including the same three species captured previously in the screening bioassay, *X. nauticus* (109 females, 104 males), *P. lecontei* (120 females, 152 males), and *P. decussatus* (three females, two males). In addition, a few *Neoclytus modestus modestus* (10 females, five males), and *Brothylus gemmulatus* LeConte (six females) also were caught during these

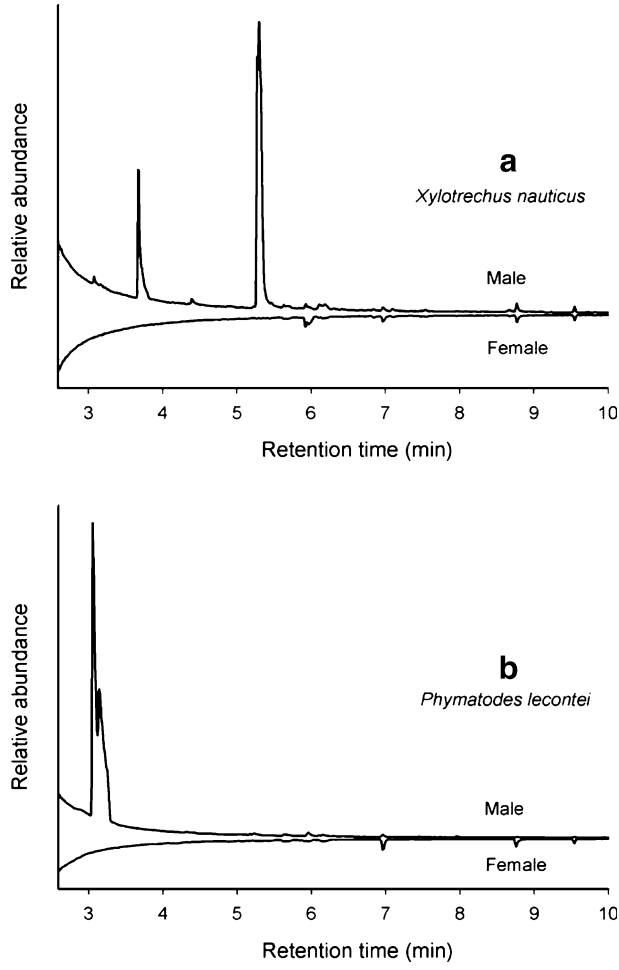


Fig. 1 Total ion chromatograms (**a**) of extracts of headspace volatiles produced by adult male (upper trace) and female (lower, inverted trace) *Xylotrechus nauticus*. The early eluting peak is 2,3-hexanedione, and the later eluting peak is a 9:1 mixture of (*R*)- and (*S*)-3-hydroxyhexan-2-one. Total ion chromatograms (**b**) of extracts of headspace volatiles produced by adult male (upper trace) and female (lower, inverted trace) *Phymatodes lecontei* (bottom). The large peak of (*R*)-2-methylbutan-1-ol in the extract from males is split because of incomplete cold trapping on the GC column during the splitless injection. An HP-5MS column was used for all analyses (see "Methods and Materials" for details)

trials. The maximum numbers of beetles caught per trap in a 24-hr period were 19 *X. nauticus* in a trap baited with generic hydroxyhexanone and 14 *P. lecontei* in a trap baited with the 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one.

Treatments differed significantly in the number of adult *X. nauticus* and *P. lecontei* that were captured (Fig. 2; sexes combined, overall ANOVA for *X. nauticus* $F_{11,\ 105}=3.67$, $P<0.001$, for *P. lecontei*, $F_{11,\ 105}=4.90$, $P<0.001$). The day effect was significant for *X. nauticus* ($P=0.005$) and *P. lecontei* ($P=0.002$). Numbers of both species captured with ethanol controls were not different from those captured with blank controls (orthogonal contrast, $P>0.65$). There was considerable variation in the numbers of beetles captured over time, with mean capture rates for the most attractive treatments (those that contained (*R*)-3-hydroxyhexan-2-one alone or in blends) ranging from 3.5±0.8 (S.E.) to 11.9±2.0 beetles of all cerambycid species per trap per day. There was no evidence that capture rates for any particular treatment were related to either air temperature or sample date (data not presented).

Mean trap catches of *X. nauticus* for all treatments that contained (*R*)-3-hydroxyhexan-2-one were significantly different from the combined controls (Fig. 2a), and were not significantly different from one another (contrast $P>0.5$). The response to (*S*)-3-hydroxyhexan-2-one was not different from the response to the controls (Fig. 2a). Sex ratios of trapped *X. nauticus* were not significantly different from 1:1 for all treatments (χ^2, $P>0.05$). Addition of 2,3-hexanedione to a 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one had no effect on trap captures (contrast $P>0.9$). The 2-methylbutan-1-ol treatment was not attractive to *X. nauticus* (Fig. 2a; orthogonal contrast *versus* controls, $P>0.7$).

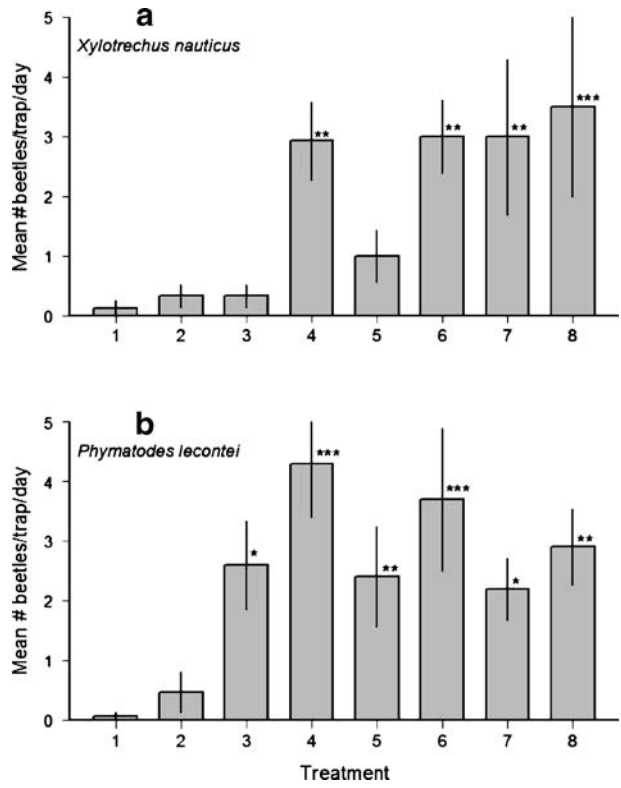


Fig. 2 Relationship between the mean number of adult *Xylotrechus nauticus* (**a**) and *Phymatodes lecontei* (**b**) captured per trap per day (sexes combined) and stereoisomeric composition of the lure. Treatments were: **1**) blank control; **2**) EtOH control; **3**) racemic 2-methylbutan-1-ol; **4**) (*R*)-3-hydroxyhexan-2-one (94% ee); **5**) (*S*)-3-hydroxyhexan-2-one (94% ee); **6**) 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one; **7**) 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one plus 2,3 hexanedione; and **8**) generic blend of all four 2,3-hydroxyhexanone stereoisomers. Error bars represent 1 S.E. ($N=15$ replicates per mean). Bars marked with asterisks are significantly different from pooled control treatments when tested with preplanned orthogonal contrasts: *, **, and *** indicate $P<0.05$, 0.01, and 0.001, respectively

Racemic 2-methylbutan-1-ol was more attractive than controls to both sexes of *P. lecontei* (Fig. 2b), suggesting that it is a male-produced aggregation pheromone component. Furthermore, all of the treatments that contained hydroxyhexanones attracted more *P. lecontei* than controls, including the (*S*)-3-hydroxyhexan-2-one treatment. Treatments that contained the (*R*)-enantiomer were not more attractive than the (*S*)-enantiomer (contrast $P > 0.2$), and the (*R*)-enantiomer was no more attractive than the 9:1 blend of (*R*)- and (*S*)-3-hydroxyhexan-2-one ($P > 0.5$), indicating no inhibition of attraction by the (*S*)-enantiomer. In addition, the (*R*)-enantiomer was no more attractive than the generic blend of hydroxyhexanones ($P > 0.1$), suggesting that the 2-hydroxyhexan-3-one isomers in the generic blend were not inhibitory. Sex ratios of beetles were female biased for the (*S*)-treatment, and male biased in the 9:1 blend treatment (Table 1), but not significantly different from 1:1 for the remaining treatments.

Too few *N. m. modestus* were captured for rigorous statistical analysis of all the treatment effects. Nevertheless, all 15 beetles were captured in traps baited with the compound that the males produced, (*R*)-3-hydroxyhexan-2-one, alone or in blends (Table 1). The strength of the response to these compounds was revealed when data from the four treatments that contained (*R*)-3-hydroxyhexan-2-one (including 9:1 (*R*)- and (*S*)-3-hydroxyhexan-2-one, with and without 2,3-hexanedione, and the generic hydroxyhexanones) were pooled and contrasted with data from the four treatments that did not contain it (blank and ethanol controls, 2-methylbutan-1-ol and (*S*)-3-hydroxyhexan-2-one treatments) and tested by chi square analysis ($\chi^2 = 15.0$; $P < 0.001$).

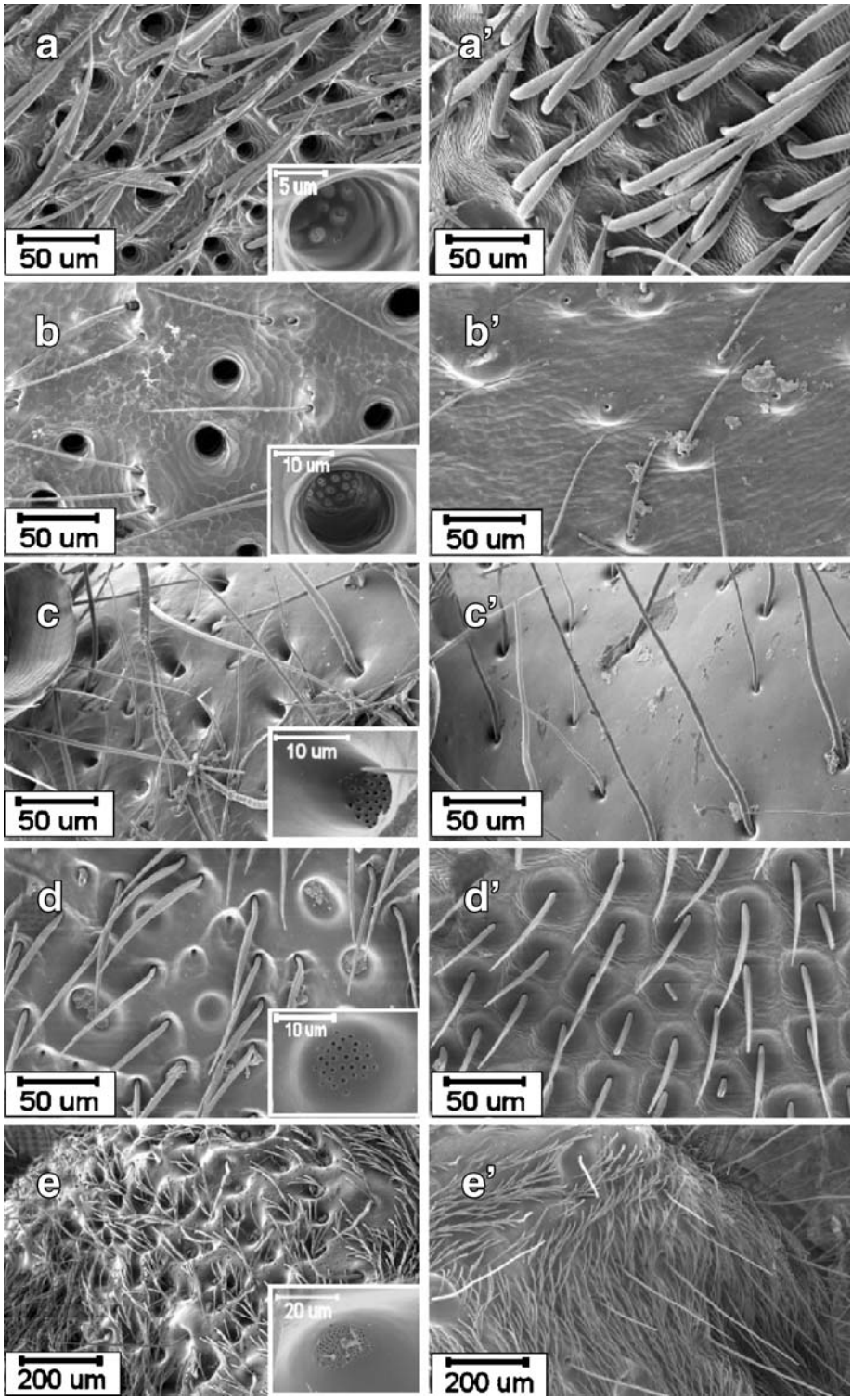
The small numbers of *P. decussatus* (also captured in the generic screening study; Table 1) and *B. gemmulatus* that were caught in the targeted bioassays prevented proper assessment of their responses to the test compounds. However, it is noteworthy that adults of these species also were caught in traps baited with (*R*)-3-hydroxyhexan-2-one, as a single component or in blends, with the exception of three *P. decussatus* caught in a trap baited with racemic 2-methylbutan-1-ol (Table 1).

A total of eight adults of the trogositid beetle *Temnochila chlorodia* were caught in traps baited with ethanol (one beetle), (*R*)-3-hydroxyhexan-2-one (two beetles), (*S*)-3-hydroxyhexan-2-one (one beetle), and generic hydroxyhexanones (four beetles), but numbers were too low to determine whether catch was caused by attraction to the lures or simply because of chance. *T. chlorodia* is a predator of scolytid beetles and is attracted by their pheromones (Bedard et al. 1980; Seybold et al. 1992; Dahlsten et al. 2004). Consequently, it seems reasonable to suppose that it also might be attracted to volatiles associated with other potential prey.

Location of Pheromone Glands

The prothoraces of males of all five of the cerambycine species that were captured had pores similar in structure to the pheromone gland pores of cerambycine species previously studied (Fig. 3; Ray et al. 2006). Pores were present on the pleura of all five species, but also on the episterna and sterna of *X. nauticus*, *P. lecontei*, *P. d. decussatus*, and *B. gemmulatus*, and the pronota of *B. gemmulatus* and *N. m. modestus*. Females had cuticular depressions that contained a single central seta and no pores (Fig. 3). Pore indentations of male *X. nauticus* and *P. lecontei* (Fig. 3a,b) were more deeply invaginated than those of the remaining species, and were much broader in *B. gemmulatus* than in species examined previously (Fig. 3e; Ray et al. 2006).

Histological sectioning of prothoraces of adult *X. nauticus* and *P. lecontei* was problematical because gut and muscle tissue did not fully solidify after infiltration and embedding. As a result, the cuticle separated from underlying tissues during sectioning. Sections of *P. lecontei*


a
5 um
50 um
a'
50 um
b
10 um
50 um
b'
50 um
c
10 um
50 um
c'
50 um
d
10 um
50 um
d'
50 um
e
20 um
200 um
e'
200 um

◀ **Fig. 3** Scanning electron micrographs of the pronotum of adult: *Xylotrechus nauticus* (**a**), *Phymatodes lecontei* (**b**), *Phymatodes decussatus decussatus* (**c**), *Neoclytus modestus modestus* (**d**), and *Brothylus gemmulatus* (**e**). For each species, male prothorax is on the left (inset, close-up of pore indentation) and female prothorax on the right (primed letters)

(not shown) were of such poor quality that no conclusions could be drawn about sexual differences in morphology. However, sections of *X. nauticus* revealed that males had a layer of cells underlying the cuticle, with cells connected to pores lying within depressions in the cuticle (Fig. 4a) that resembled the type III gland cells associated with pheromone glands of *Neoclytus a. acuminatus* F. and *N. mucronatus mucronatus* (F.) (Lacey et al. 2007a, b). This cellular layer was absent in prothoracic sections of females (Fig. 4b).

Discussion

The results of the initial field screening experiments provided support for our hypothesis that the 2,3-hydroxyketone pheromone structural motif is conserved among cerambycine species. Furthermore, this study demonstrated that species could be targeted for detailed pheromone identification by trapping live adults with generic blends of hydroxyhexanones,

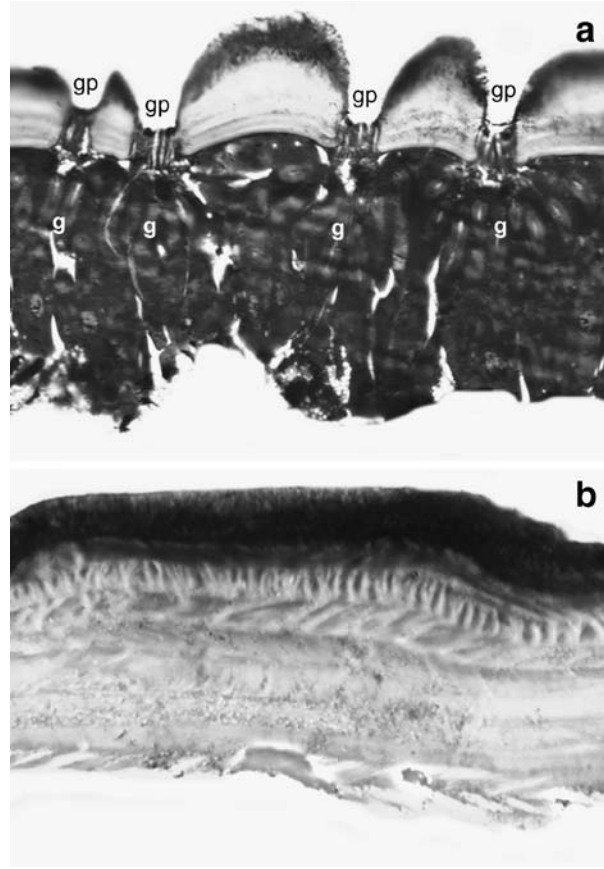


Fig. 4 Transverse histological sections through prothoraces of adult *Xylotrechus nauticus*: (**a**) male (×400; gp = gland pore; g = gland cells); (**b**) female (×400). Fig. 1

followed by collection and analysis of the volatile compounds released by the live beetles. Targeted trapping with likely pheromone components has an advantage over trapping with less selective baits such as host plant volatiles (e.g., Chénier and Philogène 1989; Sakakibara et al. 1997), because the pheromone components are likely to be much more specific to cerambycid beetles. This was borne out by the fact that our pheromone-baited traps captured only a few specimens of other types of insects, facilitating field processing of samples and collection of live beetles for pheromone identification. The selectivity of the pheromone lures may be useful in situations where pheromone-baited traps are deployed in large numbers and checked by personnel with little entomological training, as might occur during efforts to detect and delineate the range of invasive species.

The three cerambycine species that were attracted in field bioassays represent two tribes of the Cerambycinae: the Clytini (*X. nauticus* and *N. m. modestus*), and the Callidiini (*Phymatodes lecontei*; Monné and Hovore 2005). Larvae of all of these species develop in oaks (Linsley 1964), but there is scant information about their biology, life histories, or behavior (Chemsak 1963; Linsley 1964).

The structure of the pheromone components of *X. nauticus* and *N. m. modestus*, 3-hydroxyhexan-2-one, is consistent with the diol/hydroxyketone structural motif of pheromones reported for another four North American cerambycine species (see Lacey et al. 2004, 2007a). In fact, we now know from ongoing work that pheromones of several additional cerambycine species also appear to be comprised of (*R*)-3-hydroxyhexan-2-one as a single component or in combination with other structurally related compounds (Table 2). Interestingly, subsets of the species listed in Table 2 are sympatric and overlap in flight season, including most of the Californian species (Linsley 1962, 1964) and all of the Illinois species (Yanega 1996; Lacey et al. 2004). This frequent occurrence of (*R*)-3-hydroxyhexan-2-one among cerambycine species, and the response of five sympatric species to the same compound in the field trials described herein, further attest to its important role in cerambycine mating systems. The similar responses of adult *X. nauticus* to all baits that contained (*R*)-3-hydroxyhexan-2-one, including the 9:1 mixture with the (*S*) enantiomer and the generic blend of stereo- and regioisomers, suggest that the minor component, (*S*)-3-hydroxyhexan-2-one, was not necessary for attraction, and that the 2-hydroxyhexan-3-one regioisomers had no inhibitory effect for this species. In contrast, Lacey et al. (2007a) showed that *N. m. mucronatus* was more strongly attracted to (*R*)-3-hydroxyhexan-2-one than to racemic 3-hydroxyhexan-2-one, indicating that the presence of the wrong enantiomer was indeed inhibitory for that species. Furthermore, the activity of the natural pheromone of male *N. a. acuminatus*, (2*S*,3*S*)-hexanediol, was not affected by the presence of the other enantiomer, but one or both of the (2*R**,3*S**)-diastereomers were inhibitory (Lacey et al. 2004). We conclude from these limited data that certain species within communities of cerambycines may be only weakly attracted or not attracted at all to traps baited with generic lures. Nevertheless, even weak attraction may be sufficient to garner sufficient specimens for pheromone collection and identification. This is particularly true given that individual beetles produce relatively large amounts of pheromone. For example, the eight male *X. nauticus* that produced pheromone in the quantitation study yielded an average of almost 100 μg of pheromone per individual per day, with one individual producing 310 μg per day. This quantity of pheromone is sufficient for obtaining mass, GC-Fourier transform infrared (FTIR), and even nuclear magnetic resonance (NMR) spectra, as well as for microchemical tests, suggesting that it should be possible to identify even a previously unknown pheromone component from volatiles collected from a single beetle.

It must also be emphasized that it is unlikely that production of pheromone occurs at a constant rate throughout the day and night. Instead, the pheromone is probably produced

Table 2 Male Cerambycinae that produce (*R*)-3-hydroxyhexan-2-one

Tribe	Species	Relative proportion[a] (%)	Location of study	Reference
Anaglyptini	*Anaglyptus subfasciatus* Pic	>80	Japan	Leal et al. 1995
Callidiini	*Pyrrhidium sanguineum* (L.)	>90	Europe	Schröder et al. 1994
Callidiini	*Hylotrupes bajulus* (L.)	75	Europe	Fettköther et al. 1995
Clytini	*Megacyllene robiniae* (Förster)	20	Illinois, USA	Unpublished data
Clytini	*Neoclytus balteatus* LeConte	80	California, USA	Unpublished data
Clytini	*Neoclytus m. modestus* Fall	100	California, USA	Present study
Clytini	*Neoclytus mucronatus mucronatus* (F.)	100	Illinois, USA	Lacey et al. 2007a
Clytini	*Neoclytus mucronatus vogti* Linsley	100	Arizona, USA	Unpublished data
Clytini	*Sarosesthes fulminans* (F.)	>80	Illinois, USA	Unpublished data
Clytini	*Xylotrechus arvicola* (Olivier)	100	Spain	Núñez and Hall, unpublished data
Clytini	*Xylotrechus colonus* (F.)	>40	Illinois, USA	Unpublished data
Clytini	*Xylotrechus nauticus* (Mannerheim)	100	California, USA	Present study
Elaphidiini	*Anelaphus pumilus* (Newman)	100	Illinois, USA	Unpublished data
Trachyderini	*Tragidion armatum brevipenne* Linsley	100	California, USA	Unpublished data

[a] Estimated relative proportion of (*R*)-3-hydroxyhexan-2-one of the total blend of hydroxyhexanones, diols, and diones of C_6 and C_8 chain length.

over a few hours or less, during the period of daily reproductive activity typical of each species. For example, in previous studies, male beetles periodically adopted a characteristic posture during which pheromone was produced (Lacey et al. 2004, 2007b), and male *X. nauticus* also adopted this posture at intervals throughout the day. Thus, beetles actually may produce pheromones at rates of 100 μg/hr or more. This may explain the relatively large doses of synthetic pheromone (in this study, 25 mg of active compound per lure, being released at ~0.5 to 1 mg/hr) that appear to be necessary to attract significant numbers of cerambycids (e.g., Lacey et al. 2004, 2007a).

The fact that male *X. nauticus* produce an aggregation pheromone and not a sex pheromone was confirmed during our field bioassays with synthetic pheromone, in which similar numbers of males and females were attracted to baited traps. Before this study, the only cerambycid species known to produce aggregation pheromones were *N. a. acuminatus* and *N. m. mucronatus* (Lacey et al. 2004, 2007a). The fact that both sexes of their congener, *N. m. modestus*, responded to synthetic pheromone in the present study also suggests an aggregative function. Although we captured only female *B. gemmulatus*, the sample size was too small to draw any firm conclusions about the sexual specificity of its pheromone.

The attraction of both sexes of *P. lecontei* to 2-methylbutan-1-ol confirmed that it also serves as a male-produced aggregation pheromone. It is possible that adults may have responded even more strongly had we had the correct, insect-produced (*R*)-2-methylbutan-1-ol to test, rather than the racemic blend. This compound had not previously been associated with cerambycids, although it has been reported as a defensive compound for other types of beetles (Dettner and Reissenweber 1991). It also has been reported as a plant-produced attractant or kairomone for nitidulid beetles (Lin and Phelan 1991) and palm weevils (Rochat et al. 2000). Males of the cerambycid *Hylotrupes bajulus* produce the related 1-butanol (Fettköther et al. 1995), which was reported to enhance attraction of adult beetles to (*R*)-3-hydroxyhexan-2-one (Reddy et al. 2005).

The absence of (*R*) or (*S*)-3-hydroxyhexan-2-one in extracts of headspace volatiles collected from male *P. lecontei* was surprising, given the strong response of both sexes to these compounds or their blend in the field (Table 1). It is possible that male *P. lecontei* do indeed produce 3-hydroxyhexan-2-one, but not under the unnatural conditions of headspace collection in the laboratory. However, this seems unlikely, given that the male-specific compound (*R*)-2-methylbutan-1-ol was present in large amounts in those extracts. Alternatively, adult *P. lecontei* may not produce hydroxyhexanones themselves, but rather may have evolved an ability to detect these compounds for exploitation of the communication channel of other cerambycine species as a method of locating larval hosts. *Phymatodes lecontei* was not only sympatric with *X. nauticus*, but both species had the same pattern of circadian activity. *Phymatodes lecontei* undoubtedly shares hosts with *X. nauticus*, but nothing is known of the ecological interactions between these species.

The presence of pheromone gland pores on the prothoraces of male *X. nauticus*, *P. lecontei*, and *N. m. modestus* was consistent with the responses of these species to synthetic pheromone. Males of the remaining two species, *P. decussatus* and *B. gemmulatus*, also had gland pores that were missing in females. The structures of pore indentations in *X. nauticus* and *P. lecontei* were similar to the deeply invaginated indentations of *Xylotrechus quadripes* Chevrolat, and also those of the cerambycine *Achryson surinamum* (L.) (Ray et al. 2006). The shallower pore indentations and the distribution of pores in *N. m. modestus*, *P. d. decussatus*, and *B. gemmulatus* were similar to those of the congeners *N. a. acuminatus* and *N. m. mucronatus*, as well as other cerambycine species (Lacey et al. 2007b; Ray et al. 2006). The layer of glandular cells associated with prothoracic pores of male *X. nauticus* provided further evidence that these pores are the site of pheromone release.

Our capture of multiple cerambycine species at a single location over a short period of time demonstrates the potential for using generic blends of likely pheromone components in the development of tools for monitoring and control of the many cerambycine species that are pests (e.g., Solomon 1995). These generic blends can be synthesized much more quickly and cheaply than the individual regioisomers or stereoisomers, providing economical lures for screening or survey trials. This study also validates the concept of using generic blends of candidate pheromones as a lead-in method for initiating the identification of pheromone components for cerambycid beetles. Furthermore, the strong attraction of several of the study species to generic hydroxyhexanones (19 beetles of one species were caught in a single trap in 1 day) demonstrates the potential for using pheromone-baited traps as a sensitive method for detecting and monitoring specific cerambycid species.

Overall, the results reported here and analogous results from previous studies of cerambycid semiochemistry suggest numerous unanswered questions relating to the production and use of pheromones within this family. For example, given that three species were attracted simultaneously at our study site with the single pheromone component (*R*)-3-

hydroxyhexan-2-one, what mechanisms might limit cross attraction between species? Despite the fact that we found several minor components in extracts (e.g., (*S*)-3-hydroxyhexan-2-one and 2,3-hexanedione in *X. nauticus*), there was no evidence that a particular species produced one or more compounds that inhibited cross-attraction of sympatric species, as frequently occurs with minor components of the pheromone blends of other insects (e.g., scarab beetles, Leal 1999; moths, Cardé and Haynes 2004). We also found no evidence that attraction to the main component, (*R*)-3-hydroxyhexan-2-one, was increased by addition of the possible minor components (*S*)-3-hydroxyhexan-2-one and 2,3-hexanedione, that might provide a unique pheromone channel for a particular species. Furthermore, some of our study species have similar diurnal patterns of reproductive activity and exploit similar hosts, and so it seems unlikely that they are partitioning their reproductive activities in time or space.

We also do not know why male cerambycid beetles produce quantities of attractant pheromones that are several orders of magnitude larger than the amounts typically produced by other insects of similar size. For example, males of the stink bug *Acrosternum hilare* (Say) produce <1 μg of pheromone per hour (McBrien et al. 2001), and the pheromone emission rates of female lepidopterans are typically much less than 1 μg/hr (e.g., *Heliothis virescens* [F.]; Pope et al. 1982). However, pheromone production is substantial in males of arctiid moths such as *Utetheisa ornatrix* (L.), females of which assess the fitness of males by their ability to produce pheromone (Eisner and Meinwald 2003). A similar situation may occur with the cerambycid species described here, with females assessing the fitness of males by their production of pheromone.

It is also unclear why the pheromones attracted both sexes of most of our study species. Typically, insects are thought to produce aggregation pheromones when there is a benefit that will accrue to all signaling and responding individuals, such as the assembly of a critical mass of bark beetles to overwhelm defenses of host trees (Schlyter and Birgersson 1999), or the aggregation of chemically defended aposematic individuals (e.g., Al Abassi et al. 1998). However, in the case of the cerambycid species in this study, there is no obvious benefit to aggregation with regard to attack of hosts because the beetles use dying or dead hosts (Linsley 1962, 1964). Furthermore, *X. nauticus* and *P. lecontei* apparently are not aposematic (Linsley 1962, 1964) and have no obvious chemical defenses, so it seems unlikely that they would aggregate for mutual defense. However, production of large quantities of pheromone is costly, and aggregation may be of selective advantage if "satellite males" assembling in the vicinity of conspecific males can intercept responding females (see Moore et al. 1995).

As this research continues, we hope to answer some of these questions and expand our understanding of the role of semiochemicals as mediators of reproductive behaviors within this diverse insect family. In particular, a more detailed understanding of taxonomic patterns of pheromone chemistry and pheromone use would be tremendously valuable for facilitating the identification of pheromones of invasive cerambycine species as they inevitably arrive in North America (see Haack 2006) or other areas of the world. This information in turn would provide the means for developing methods for detecting, controlling, and even potentially eradicating invasive species.

Acknowledgments We thank Frank Hovore (deceased), Douglas Yanega, and Ian Swift for assistance with identification of beetle species, Scott Robinson for help with SEM and histological sectioning work, Adam Martinsek for assistance with the statistical analysis, James Nardi for access to microscopy equipment, and Mariana Krugner and Ian Wright for technical assistance. We thank Carole Bell and Santa Rosa Plateau Reserve, Riverside Co., California, for access to field sites. The SEM and histology research was made

possible through collaboration with Bugscope, The Imaging Technology Group, Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign (http://bugscope.beckman.uiuc.edu/). This project was supported by funds from the Exotic/Invasive Pests and Diseases Research Program, University of California, under USDA-CSREES Grant No. 2004-34439-14691, the Alphawood Foundation of Chicago, and Hatch Project #CA-R*ENT-5181H to JGM.

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J Chem Ecol (2007) 33:909–921
DOI 10.1007/s10886-007-9268-3

Support for (*Z*)-11-Hexadecanal as a Pheromone Antagonist in *Ostrinia nubilalis*: Flight Tunnel and Single Sensillum Studies with a New York Population

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Received: 4 October 2006 / Accepted: 16 February 2007 /
Published online: 29 March 2007
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Abstract The flight-tunnel response of male Z-strain European corn borer moths (ECB), *Ostrinia nubilalis*, from a population in New York State (USA), was significantly antagonized by addition of 1% (*Z*)-11-hexadecanal (Z11-16:Ald) to their sex pheromone (a 97:3 mix of (*Z*)- and (*E*)-11-tetradecenyl acetate [Z/*E*11-14:OAc]). The level of antagonism was equivalent to that observed for the previously identified ECB antagonist, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), and supports a recent report showing that Z11-16:Ald, a minor pheromone component of the Noctuid moth, *Sesamia nonagrioides*, caused antagonism of ECB pheromone communication in sympatric populations in the Iberian Peninsula. Single-sensillum recordings from ECB antennae, which included cross-adaptation experiments, showed that the same olfactory receptor neuron processing Z9-14:OAc inputs was responsible for detecting Z11-16:Ald, and that this neuron was not responsive to two other aldehydes, (*Z*)-9-tetradecanal (Z9-14:Ald) and (*Z*)-9-hexadecanal (Z9-16:Ald), found in other moth sex pheromones. Our results show that the antagonism is not confined to one geographic region, is specific for Z11-16:Ald, and that antagonist pathways might have the potential for processing a number of structurally similar compounds.

Keywords Sex pheromones · European corn borer · *Ostrinia nubilalis* · Mediterranean corn borer · *Sesamia nonagrioides* · Behavioral antagonist · Z11-16:Ald · Flight tunnel · Single sensillum recordings · Cross adaptation

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Introduction

Mate location in many insect species is mediated by female-released sex pheromones. In almost all cases, these have proven to be mixtures, with blend quality and quantity providing for varying degrees of signal specificity (Linn and Roelofs 1995). Signal specificity is also facilitated by sensitivity to pheromone components of related species that function as behavioral antagonists (Cardé and Haynes 2004). When added in very small amounts to the sex pheromone of a particular species, the new mixture results in arrestment of pheromone-mediated flight. For example, different pheromone strains of the European corn borer moth (ECB), *Ostrinia nubilalis* (Crambidae), utilize mixtures of (*Z*)-11- and (*E*)-11-tetradecenyl acetates (*Z*11/*E*11-14:OAc) in their sex pheromones (Roelofs et al. 1985, 1987). However, males are also sensitive to a non-pheromonal compound, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), and possess a separate antennal olfactory pathway for processing this compound (Hansson et al. 1987). As little as 1% Z9-14:OAc added to the Z/E11-14:OAc mixture dramatically reduced the number of males completing upwind oriented flight in a wind tunnel (Glover et al. 1989).

A study by Eizaguirre et al. (2002) showed, in pheromone mating disruption tests for the Mediterranean corn borer, *Sesamia nonagrioides* (Noctuidae), that larval counts were significantly reduced for the target species and for ECB. Combining pheromone blends of both species in the same dispenser showed further that there was mutual antagonism for both species. Subsequently, Gemeno et al. (2006) showed that the antagonism was caused specifically by addition of (*Z*)-11-hexadecanal (Z11-16:Ald) to the ECB pheromone, with as little as 1% reducing upwind flights by 83% and trap capture by 90%. The compound Z11-16:Ald is a minor pheromone component of *S. nonagrioides* and is not part of the ECB pheromone. Here, we provide results of flight tunnel and single-sensillum recording experiments to address the following questions: 1) are ECB from a population in New York State antagonized by addition of Z11-16:Ald to their pheromone blend? 2) is the sensory input for Z11-16:Ald on the same olfactory receptor neuron (ORN) as for Z9-14:OAc?, and 3) do other aldehyde components, such as Z9-14:Ald and Z9-16:Ald, that are pheromone components in several heliothine moth species (Lee et al. 2006), also act as antagonists for ECB?

Methods and Materials

Insects A colony of the univoltine Z-strain of ECB from New York State was maintained in a walk-in environmental chamber. The Z-strain was used in our experiments because it is the predominant population in the Iberian Peninsula, the origin of moths used in the studies by Eizaguirre et al. (2002) and Gemeno et al. (2006). Mating and larval rearing conditions were as described in Roelofs et al. (1987) at a constant 25°C, 16:8 L/D photoperiod. Pupae were sexed, and the male pupae were placed on a layer of vermiculite in plastic and screen emergence cages inside a walk-in environmental chamber (25°C; 16:8 L/D photoperiod). Cages of adults were separated daily so that individuals of known age could be used for flight tunnel tests.

Chemicals Z11-14:OAc, E11-14:OAc, and Z9-14:OAc were obtained from the Pherobank http://www.pherobank.nl). Z11-16:Ald, Z9-14:Ald, and Z9-16:Ald were obtained from Bedoukian Research, Inc. (Danbury, CT, USA). Purity of each compound was verified as >95% by gas chromatography (GC). Stock solutions were prepared in high-performance

liquid chromatography-grade hexane (HPLC-grade hexane). Solutions were applied to red rubber septa (Thomas Scientific, Swedesboro, NJ, USA; Glover et al. 1989) for flight-tunnel tests, and to filter paper strips for electrophysiological measurements (see below).

Flight Tunnel Adults were tested in the sustained-flight tunnel during their second to third night as adults, under standard conditions used in previous studies of *O. nubilalis* (Glover et al. 1989): 20–21°C, 60–65% relative humidity, 0.50 m/sec air flow, and illumination of 11 lx of red light at the tunnel floor, during the third to the sixth hour of scotophase. Adults were taken to the room housing the flight tunnel 1 hr before the start of scotophase and placed individually in screen release cages so they could acclimate to the flight tunnel room environment. The temperature during the 1-hr period of acclimation, was 25°C, then dropped after lights-off to 20–21°C. Adult moths were tested individually, and males were scored for three behaviors in the flight sequence: taking flight (TF) from the release cage; initiation of upwind flight (UP) a minimum of 20 cm toward the source; and source (SC) contact after completing upwind flight to the rubber septum.

Two experiments were conducted to study the effects of Z11-16:Ald on ECB pheromone-mediated flight behavior. The first was designed to determine whether Z11-16:Ald arrested the upwind flight behavior of males, and to compare the level of antagonism with Z9-14:OAc (Glover et al. 1989). Males were tested to four treatments: 1) the Z-strain 97:3 Z/E11-14:OAc blend (30 μg); 2) the Z-strain blend (30 μg)+1% Z9-14:OAc; 3) the Z-strain blend (30 μg)+1% Z11-16:Ald; and 4) the Z-strain blend (30 μg)+10% Z11-16:Ald. The second experiment was designed to determine the specificity of the antagonist effect of Z11-16:Ald by testing two additional, structurally related aldehydes found in other pheromone systems, Z9-14:Ald and Z9-16:Ald (Lee et al. 2006). Males were tested to three treatments: 1) the Z-strain 97:3 Z/E11-14:OAc blend (30 μg); 2) the Z-strain blend (30 μg)+10% Z9-14:Ald; and 3) the Z-strain blend (30 μg)+10% Z9-16:Ald. Over the course of both experiments, 30 males were tested to each treatment. Males were tested only once, with each of the treatments of an experiment used on each day of testing.

For each flight tunnel experiment, the number of moths in each treatment that exhibited each behavior in the upwind flight sequence was combined for all testing periods and converted to a percentage value for graphical display. *Post hoc* pairwise comparisons with Fisher's exact tests were implemented according to the JMP statistical analysis program for Macintosh ($P<0.05$).

Electrophysiology With excised antennae, ORN recordings were made by using the cut sensillum technique (Kaissling 1974; van der Pers and den Otter 1978). The basal end of each antenna was placed in a saline-filled electrode and positioned with a micromanipulator such that a single trichoid sensillum would be cut between a vertical tungsten knife and horizontal glass knife. The cut sensillum was then contacted with a saline-filled glass micropipette containing a Ag recording electrode. The AC signal passed through the built-in amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) of a portable recording unit into a computer and was recorded with Syntech software (Autospike v.32; Syntech, Kirchzarten, Germany).

A stream of purified, humidified air blew continuously over the antenna (10 ml/sec) through a 25-cm long glass tube (8 mm ID), the end of which was placed 2 cm from the antenna. With a stimulus flow controller device (SFC-2, Syntech), a 50-msec air pulse at 40 ml/sec flow rate was injected through the odor cartridge and into the air stream. Linear flow through the air stream was ~0.3 m/sec. Cartridges were created from dilutions (0.1, 1, and 10 μg/μl) of Z11-16:Ald and Z9-14:OAc, and from 10 μg/μl of Z11-14:OAc, E11-14:

OAc, Z9-14:Ald, and Z9-16:Ald in HPLC-grade hexane. For each concentration, 10 μl was pipetted onto a 0.5×2.0 cm filter paper strip held in a 15-cm-long Pasteur pipette odor cartridge. The filter paper loadings thus included 1, 10, or 100 μg for the various compounds.

By using 24 antennae from 18 moths, dose–response curves were obtained for Z11-16:Ald and Z9-14:OAc. A balanced design with respect to the order of application of treatments was employed to allow a more accurate comparison of the relative responsiveness of the ORNs to these compounds. For the first 12 antennae, recordings were performed with 1, 10, and 100 μg loadings of Z11-16:Ald in the first sensillum, whereas recordings to the same range of dosages of Z9-14:OAc were performed on a second sensillum. For the remaining 12 antennae, Z9-14:OAc was used before Z11-16:Ald. At least 30 sec was allowed to elapse between all stimulations. Spikes were counted from within 300 msec of the initiation of neuronal activity, which can occur between 50 and 200 msec after the stimulus is first applied (Fig. 1a). As previously reported, there was little spontaneous background activity (Cossé et al. 1995; Domingue et al. 2006), and the initiation of response was easily discerned by the first appearance of spikes. Analysis of variance (ANOVA) was performed by using the GLM procedure of SAS (Version 9.1), to test the effects of order of application and compound on response at the 100 μg loading. Responses at 1 μg were nearly always zero for both compounds. Responses at 10 μg to both compounds often occurred, but there were enough non-responses to cause non-normality of the data at this concentration (Wilks normality, α=0.05). However, by using the increase in spike frequency from 10+ to 100 μg, which is normally distributed, we performed a similar ANOVA.

Paired stimulations aimed at exploring cross-adaptation were also performed, in most cases by using additional sensilla from the antennae that were used to obtain dose–response curves. Air from two pheromone cartridges, with loadings of 100 μg, was pulsed as

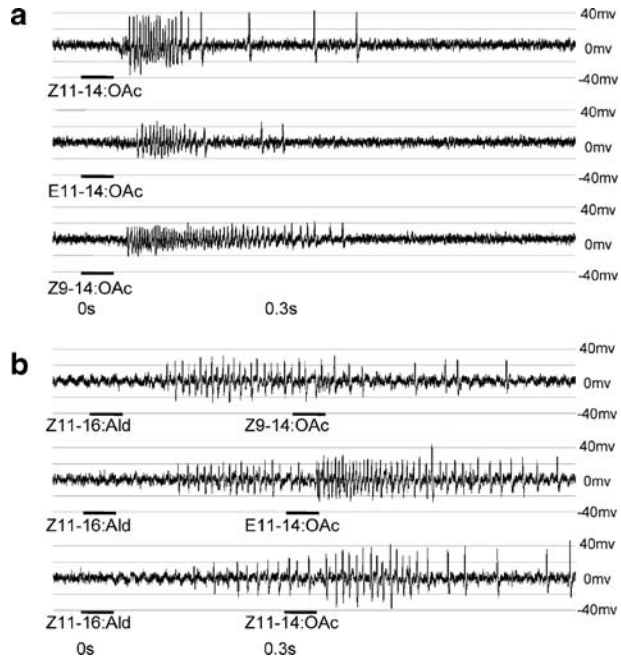


Fig. 1 Examples of ORN stimulation in Z-strain ECB in two sensilla from different individuals, using 100-μg loadings in each case: (a) Spike patterns in response to the known pheromone components Z11-14:OAc and E11-14:OAc and the antagonist Z9-14:OAc. (b) Cross-stimulation involving Z11-16:Ald with the antagonist Z9-14:OAc, the minor pheromone component E11-14:OAc, and the major pheromone component Z11-14:OAc. Horizontal bars indicate stimulus application and duration

described above, with an interval of 300 msec between the start of the pulses from the first and second cartridges. This method is similar to that employed for other moths (Ochieng et al. 2002) and was calibrated from unpublished data for this species such that for each ORN there is self-adaptation, marked by a lack of repeated stimulation by the same compound. To infer which ORN is sensitive to Z11-16:Ald, cartridge pairs used for cross-adaptation *versus* this compound included, Z9-14:OAc, Z11-14:OAc, and E11-14:OAc, which are, respectively, known to stimulate distinct ORNs (Hansson et al. 1987). It was readily apparent that cross-adaptation occurs between Z11-16:Ald and Z9-14:OAc. For comparative purposes, and to ensure the validity of our experimental technique, we also performed self-adaptation with Z9-14:OAc and Z11-16:Ald.

At least 10 replicates of all paired stimulations were performed. No more than three paired stimulations were performed per sensillum, with at most five sensilla examined per antenna. In many cases, there were two distinct bursts of neuronal activity with obviously different spike sizes (Fig. 1b). To assess the intensity of response to stimulation by the first compound, spikes were counted within 300 msec of the initiation of neuronal activity. For the second compound, initiation of the second response was noted if a different spike size from the first stimulus appeared, or if there was a period of inactivity of greater than 50 msec, which would normally not be observed in spike trains arising from a single stimulation. In a few rare cases, there were similarly sized action potentials in a continuously uninterrupted pattern for more than 300 msec after the initial response. Because the initiation of a second response was unclear in these cases, we assigned the counts for the first and second compounds to the respective periods between 0 and 300 msec and 300 and 600 msec after the first onset of neuronal activity.

To describe the process of cross-adaptation statistically we performed three separate ANOVA, considering pairings involving: (1) Z11-16:Ald by E11-14:OAc cross-adaptation; (2) Z11-16:Ald by Z11-14:OAc cross-adaptation: and (3) Z11-16:Ald by Z9-14:OAc cross-adaptation or self-adaptation of either compound. For this analysis, we discarded categorization of spike size and looked at the effect on spike frequency of being the first or second compound applied (Order), which would indicate adaptation. For all three analyses, the effect on spike frequency of compound identity (Compound) and the interaction between Compound and Order were also considered. For the third analysis only, the effect of cross-adaptation vs. self-adaptation (X-adapt) was also considered, along with its interactions with Compound and/or Order.

To explore the sensitivity of Z-strain ECB ORNs to the aldehydes, Z9-14:Ald and Z9-16:Ald, an additional experiment was performed. Responses to Z9-14:Ald and Z9-16:Ald were assessed in comparison to a control consisting of Z11-16:Ald or Z9-14:OAc, with 24 antennae from different moths. In all cases, 100 μg cartridge loadings were used. On four successive sensilla from each antenna, one of the following sequences of stimulations was performed: A) Z9-14:Ald, Z9-16:Ald, and Z11-16:Ald; B) Z9-14:Ald, Z9-16:Ald, and Z9-14:OAc; C) Z9-16:Ald, Z9-14:Ald, and Z11-16:Ald; D) Z9-16:Ald, Z9-14:Ald, and Z9-14:OAc. Across 24 moths, each of the possible combinations of treatments A–D, with respect to sensillum order, was completed once. With this design, Z9-14:Ald and Z9-16:Ald were tested 96 times, and Z11-16:Ald and Z9-14:OAc 48 times. For this last experiment, we examined both the spike frequency and presence or absence of response for each test compound and the control with respect to sensillum order. For a small number of pairwise comparisons of interest, *t* tests or Fisher's exact tests were used depending on whether the spike frequency or presence of response was considered. Similarly, least-square or logistic regressions were performed when appropriate for either type of variable, by using Proc Reg or Proc Logistic in SAS.

Results

Flight Tunnel Experiment 1: There was no difference in the percentage of males taking flight (100%) among the four treatments (Z-strain blend alone,+1% Z9-14:OAc,+1% or 10% Z11-16:Ald, Fig. 2a). Significant decreases, however, were observed in the percentage of males initiating upwind flight with Z-strain blend and any of the added compounds, compared with the Z-strain blend alone. The greatest decrease occurred with 10% Z11-16:Ald treatment (0% UP vs. 100% for Z-strain alone; Fisher's exact $P<0.001$, $df=1$), followed by 1% Z9-14:OAc and 1% Z11-16:Ald (62 and 74%, respectively, vs. 100% for Z-strain alone; Fisher's exact $P<0.001$, $df=1$, for 100% vs. 74%). Significant decreases were further observed in the percentage of males reaching the source, with 0, 4, and 8% SC for 10% Z11-16:Ald, 1% Z9-14:OAc, and 1% Z11-16:Ald, respectively, compared with the Z-strain blend alone (97% SC; Fisher's exact $P<0.001$, $df=1$, for 100% vs. 8%).

Experiment 2: As with experiment 1, male Z-strain ECB exhibited high levels of upwind flight (UP, 100%) and source contact (SC, 96%) with the Z-strain blend alone (Fig. 2b). However, addition of 10% Z9-14:Ald or Z9-16:Ald did not affect the percentage of upwind

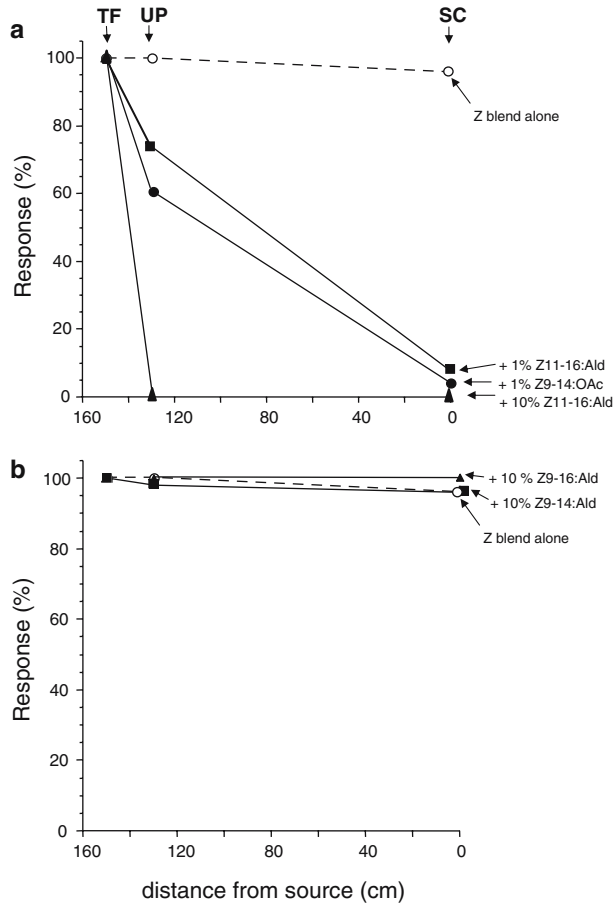


Fig. 2 Proportion of male ECB of the Z-strain exhibiting three behaviors in flight tunnel tests (taking flight, TF; upwind flight, UP; source contact, SC) to: (a) the Z-strain 97:3 Z/E11-14:OAc blend alone (30 μg), the Z-strain blend+ 1% or 10% Z11-16:Ald, or the Z-strain blend+1% Z9-14:OAc; (b) the Z-strain 97:3 Z/E11-14:OAc blend alone (30 μg), the Z-strain blend+10% Z9-14:Ald or 10% Z9-16:Ald. $N=30$ for each of the treatments. See text for statistical comparisons

flights and source contact observed with the Z-strain blend alone (100% SC with Z9-16:Ald, 96% SC with Z9-14:Ald).

Electrophysiology We were able to obtain dose–response curves to both Z11-16:Ald and Z9-14:OAc (Fig. 3a). From Fig. 3, it appears as if the sensilla are relatively more sensitive to Z11-16:Ald if it is applied first. However, statistical analyses did not confirm the significance of this pattern. At 100 μg, responses to Z9-14:OAc were greater than those to Z11-16:Ald (i.e., Compound is significant, Table 1). This result was not significantly influenced by which compound was applied first (i.e., Order and the interaction, Table 1). Similarly, for the increase in spike frequency between the 10 to 100 μg loadings, the effect of Compound was significant, but not Order or the interaction (Table 1).

In a sensillum not used for any statistical analysis (Fig. 1a), we demonstrated that ORN responses to Z11-14:OAc, E11-14:OAc, and Z9-14:OAc occurred with the same relative spike sizes as found previously (Hansson et al. 1987; Roelofs et al. 1987). It was apparent in cross-adaptation experiments that, based on spike size, Z11-16:Ald stimulated the same ORN as did Z9-14:OAc. This cross-responsive (i.e., cross-adapted) ORN was usually characterized by the smallest spike size (Fig. 1b). The ORN responding to E11-14:OAc was most often of intermediate spike size, being slightly smaller than that for Z11-14:OAc, which was always the largest (Fig. 1b).

These visual observations were supported by statistical analysis of the cross-adaptation data. When Z11-16:Ald was paired with E11-14:OAc or Z11-14:OAc, there was no effect of Order on spike frequency (Table 2, Fig. 4). Analysis of spike frequencies in stimulations involving Z11-16:Ald and Z9-14:OAc showed a strongly significant effect of Order (Table 2, Fig. 4), meaning that the second stimulation was generally inhibited by the first. In this analysis, there was also a significant effect of Compound (Table 2) demonstrating

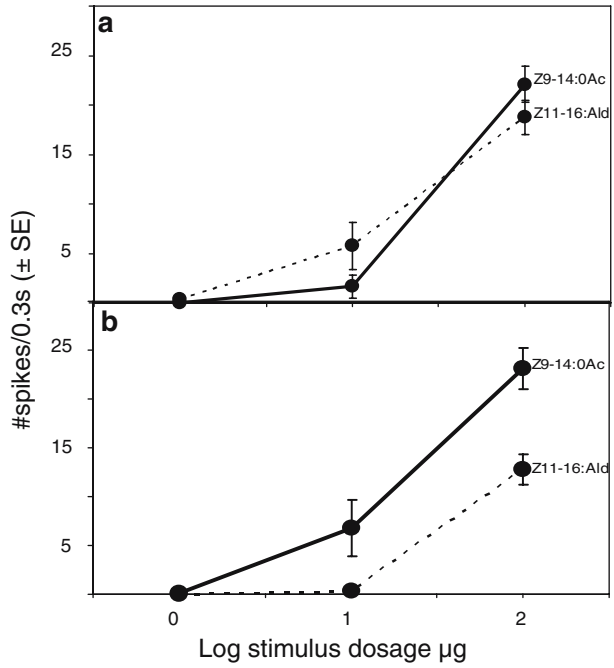


Fig. 3 Dosage-dependent response of male Z-strain ECB ORNs to Z11-16:Ald and Z9-14:OAc. (a) For $N=12$ antennae, dose-response testing was performed on the first sensillum to Z11-16:Ald, and on a second sensillum to Z9-14:OAc. (b) For another $N=12$ antennae, dose-response was performed first to Z9-14:OAc, and then to Z11-16:Ald

Table 1 Analysis of variance of spike frequency (spikes/0.3 sec) at a 100-μg loading and spike frequency increase from a 10- to 100- μg loading, both with respect to whether Z9-14:OAc or Z11-16:Ald is employed, and in which respective order per antenna these compounds were tested

Variable	Effect	*df*	*MS*	*F*	*P*
Spikes at 100 μg	Order	1	75.00	1.34	0.260
	Compound	1	560.33	9.98	0.005
	Order–Compound	1	147.00	2.62	0.120
	Error: Antenna (Order)	22	56.121		
Spike frequency increase (10 to 100 μg)	Order	1	65.33	0.84	0.370
	Compound	1	38.33	4.94	0.037
	Order–Compound	1	36.75	0.47	0.500
	Error: Antenna (Order)	22	78.07		

Order distinguishes whether the response was the first or second on an antenna. Compound distinguishes whether Z9-14:OAc or Z11-16:Ald is employed for a given stimulation. For both cases, we use the Antenna for the model error, which is nested in the specific Order treatment.

that over all treatments, the ORNs were more sensitive to Z9-14:OAc than to Z11-16:Ald. However, there was no significant effect of self- *versus* cross-adaptation (X-adapt) or any interaction effect (Table 2).

Responses by the smallest-spiking (antagonist) ORN to two other aldehydes, Z9-14:Ald and Z9-16:Ald, were generally negligible in single stimulations, yielding at most 2.4 spikes per 300 msec when Z9-14:Ald was puffed on the first sensillum of a particular antenna (Fig. 5a). It did not appear that these responses occurred in the E11- or Z11-14:OAc-tuned ORNs. On the first sensillum, when either Z9-14:Ald or Z9-16:Ald had equal opportunity to be puffed first, there was a 25% success rate of getting a response to Z9-14:Ald, (Fig. 5b).

Table 2 Analysis of variance of spike frequency (spikes/0.3 sec) for the cross-stimulation experiments grouped by the pairing of compounds with respect to Z11-16:Ald

Experiment	Effect	*df*	*MS*	*F*	*P*
E11-14:OAc vs. Z11-16:Ald	Order	1	0.319	0.01	0.935
	Compound	1	1139.147	24.26	<0.001
	Order–Compound	1	195.669	4.03	0.054
	Error: Pair(Order)	21	46.92		
Z11-14:OAc vs. Z11-16:Ald	Order	1	27.225	0.56	0.462
	Compound	1	133.225	2.76	0.114
	Order–Compound	1	0.225	0.00	0.946
	Error: Pair(Order)	18	48.225		
Z9-14:OAc vs. Z11-16:Ald	X-adapt	1	173.852	2.50	0.121
	Order	1	7896.071	113.72	<0.001
	Compound	1	775.013	11.16	0.002
	X-adapt–Order	1	20.885	0.30	0.586
	Order–Compound	1	49.613	0.71	0.403
	X-adapt–Compound	1	78.013	1.12	0.295
	Error: pair (X-adapt–Order)	42	69.437		

Order distinguishes whether a stimulus was first or second in the paired stimulation. Compound distinguishes which stimulus was provided for a given stimulation. X-adapt distinguishes whether a stimulus occurred within a cross-stimulated pair, or if the same stimulus was repeated. In each case, we use the unit of the unique paired stimulation event, Pair, as the error, which is nested in the specific Order or X-adapt treatment performed.

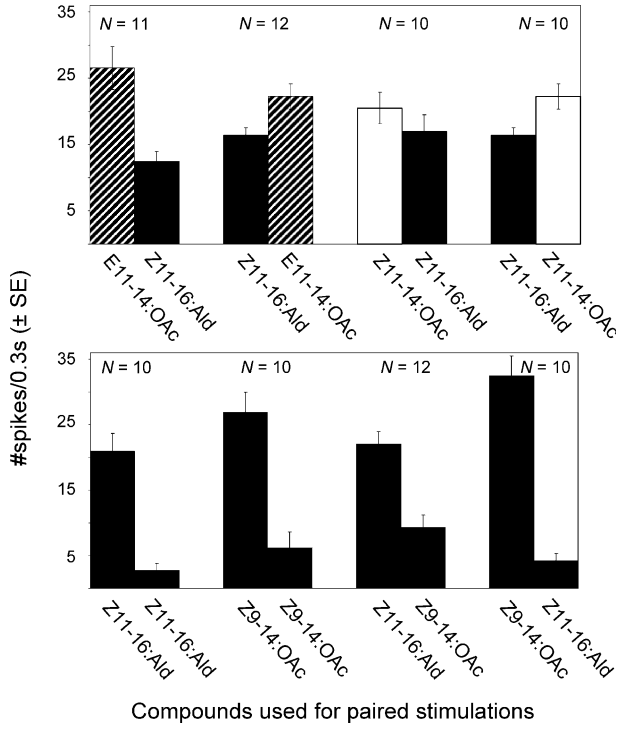


Fig. 4 Number of spikes per 300 msec for cross-stimulation involving Z11-16:Ald, Z11-14:OAc, E11-14:OAc, and Z9-14:OAc at 100-µg loadings. Differences in shading indicate ORNs with distinct spike sizes: a large spike size for Z11-14:OAc, an intermediate spike size for E11-14:OAc, and a small spike size for Z11-16:Ald and Z9-14:OAc

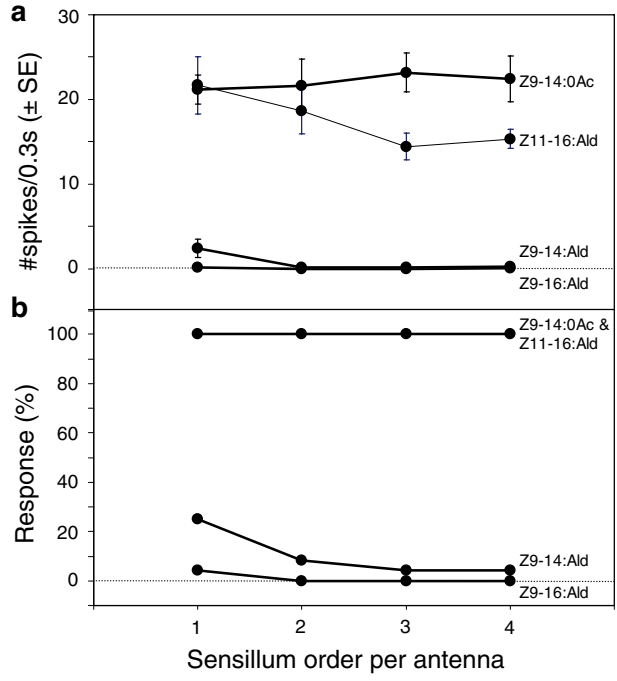


Fig. 5 Sensitivity of Z-strain ECB ORNs to Z9-14:Ald, Z9-16:Ald, Z11-16:Ald, and Z9-14:OAc in each successive sensillum tested per antenna, measured with respect to: (a) the mean (± SE) number of spikes stimulated over 300 msec at 100-µg loadings and (b) the percentage of sensillae responding. For Z9-14:Ald and Z9-16:Ald N=24 for each data point, whereas for Z11-16:Ald and Z9-14:OAc N=12

This is statistically different from the 100% response to the control compounds (Fisher's exact test, $P<0.001$). Under the same conditions, there was only a 4% success rate of this ORN responding to Z9-16:Ald on the first sensillum, which is marginally different (Fisher's exact test, $P=0.098$) from the 25% rate for Z9-14:Ald (Fig. 5b). Logistic regression for likelihood of response to Z9-14:Ald showed a significant effect of sensillum order for Z9-14:Ald ($P=0.026$), but not Z9-16:Ald ($P=0.918$).

Although designed primarily to explore the relative sensitivity of the antagonistic ORN to Z9-14:Ald and Z11-16:Ald, the final experiment also provided the opportunity to examine how the response to Z11-16:Ald and Z9-14:OAc changed with the use of multiple sensilla per moth. There was no significant difference between spike frequency for the two compounds at the first sensillum ($P=0.889$). However, a least-squares regression for Z11-16:Ald, had a significant negative slope ($P=0.031$), whereas the slope for Z9-14:OAc was not different for a similar regression involving Z9-14:OAc ($P=0.633$).

Discussion

Effects of Z11-16:Ald on Male ECB Flight Behavior Our results with males from a New York state ECB population support the finding by Gemeno et al. (2006) that Z11-16:Ald, when added to the ECB pheromone blend, can significantly arrest upwind flight behavior of male ECB. Addition of 1% Z11-16:Ald to the ECB Z-strain 97:3 Z/E11-14:OAc mixture reduced the proportions of males initiating upwind flight by 25% and the proportion reaching the source by 88%. These values compare well with the 26 and 83% reductions observed in flight tunnel tests reported by Gemeno et al. (2006). Our results show further that the antagonism by Z11-16:Ald is not restricted to the area of the Iberian Peninsula where *O. nubilalis* and *S. nonagrioides* are in sympatry, but also extends to ECB populations in North America. A third finding from our studies is that the effect appears to be specific to Z11-16:Ald because the related Z9-14:Ald and Z9-16:Ald, known pheromone components of other moth species, did not affect male flight behavior.

The effect of Z11-16:Ald was characteristic of other known pheromone behavioral antagonists (Linn and Roelofs 1995), evidenced by (1) a pronounced reduction in behavioral response when very low levels (1%) of the compound were added to the sex pheromone, and (2) significant reductions in the number of males completing upwind flights to the pheromone source (SC in Fig. 2). As for other behavioral antagonists, there was less of an effect on earlier behaviors such as taking flight or initiating upwind flight in ("locking onto") the pheromone plume (Fig. 2). Our anecdotal observations, with the 1% Z11-16:Ald treatment, showed that males typically exhibited upwind zig-zag flights, characteristic of pheromone-mediated flight, from one-half to two-thirds of the distance to the source, then began casting across wind, or hovered narrowly in place for several seconds, before exiting the pheromone plume. This flight pattern has been characterized as "arrestment" of upwind flight and is typical of the effect of behavioral antagonists (Baker 1989).

Olfactory Neuron Responses to Z11-16:Ald Gemeno et al. (2006) reported results from EAG experiments suggesting that Z11-16:Ald was not interacting with ORNs specific for the ECB pheromone components. EAG cross-adaptation experiments, however, did suggest the possibility that Z11-16:Ald and Z9-14:OAc were detected by the same ORN, one that previously had been shown to be specific for the latter compound (Hansson et al. 1987). In our study, single-sensillum recordings confirmed, via spike amplitude-matching, that a type

of ORN within the trichoid sensilla on ECB antennae is tuned to respond to both the Z11-16:Ald behavioral antagonist (Gemeno et al. 2006; this paper) and another antagonist Z9-14:OAc (Glover et al. 1989). There was negligible activity from this ORN in response to Z9-14:Ald or Z9-16:Ald, both of which were behaviorally inert. That the responses to Z11-16:Ald occurred on the same ORN that detects the presence of Z9-14:OAc was also supported by cross-adaptation studies.

(Z)-11-Hexadecanal and Pheromone Behavioral Antagonism in ECB: Adaptation or Fortuitous Mimic? The importance of behavioral antagonists in pheromone communication systems is well established (Cardé and Haynes 2004). These compounds contribute to signal specificity by lowering the probability of cross-attraction between species that utilize common pheromone components. This antagonism might involve closely related species that have the potential to mate or species that are more distantly related. In either case, signal specificity is enhanced in a "noisy" chemical environment (Linn and Roelofs 1995). In most of the known cases, the antagonist chemicals are components involved in communication channels used by species that share common pheromone components, as a result of common lineage and use of similar biosynthetic pathways. Thus, for ECB, Z9-14:OAc is a potential by-product from its pheromone biosynthetic pathways and is also a pheromone component in two related *Ostrinia* species in Japan (*O. zealis* and *O. zaguliaevi*, Ishikawa et al. 1999). Phylogenetic analysis suggests that the latter species are ancestral to the group including ECB (Kim et al. 1999). Therefore, divergence might have involved a change in functionality for Z9-14:OAc. A second, well-studied example involves the heliothine moths, *Heliothis virescens* and *Helicoverpa zea* (Vickers et al. 1998), which utilize the same major pheromone component, Z11-16:Ald. In *H. virescens*, the compound Z9-14:Ald is a pheromone component, whereas in *H. zea* it acts as an antagonist. In both of these examples, males possess a separate, specialized, sensory pathway to process the antagonist input, suggesting that there has been selection for the character to enhance reproductive isolation or interference between species sharing common components (Löfstedt 1993; Linn and Roelofs 1995; Cardé and Haynes 2004).

The examples above illustrate the point that changes in odorant receptor (OR) expression or functionality of components in a common pathway are mechanisms that could give rise to new pheromone component blends, or behavioral antagonists. Antagonism could, however, also involve a chemical that is not a member of the suite of compounds that a group of species uses for its pheromones, if that chemical binds to an OR of an established antagonist pathway. We propose that this might be the case with Z11-16:Ald and ECB. This conclusion stems firstly from the observation that Z11-16:Ald is not involved in any known sex pheromone in an *Ostrinia* species (Ishikawa et al. 1999), and second, the aldehyde is structurally similar to Z9-14:OAc with respect to chain length and position of the double bond relative to the carbonyl group and the hydrocarbon end. Both behavioral antagonists might, therefore, bind to the same OR expressed in the ORN involved in antagonism, resulting in significant firing and a reporting of their presence to higher centers via the antagonist pathway. This explanation allows for the possibility that a small number of structurally similar compounds might exert an effect without having been under selection pressure to interfere with sex pheromone communication of two species sharing the same habitat. Gemeno et al. (2006), in fact, raised caution about the adaptive significance of their finding with respect to habitat-specific interactions between ECB and *S. nonagrioides*, noting that the antagonistic effect of Z11-16:Ald was initially observed as a result of combining pheromone blends in field trapping and pheromone mating disruption tests. The two species utilize the same host plants and have seasonal overlap, but are in different

families, differ in size, have no shared pheromone components, and mate at different times in the scotophase. In this scenario, the antagonist effect of Z11-16:Ald could be considered a coincidence of chemical structural similarity.

Alternatively, two different pheromone ORs could be expressed on the same dendrite, with one specifically binding Z9-14:OAc and the other, Z11-16:Ald. Although pheromone ORNs have generally been thought to express only one OR gene, recent studies on *Drosophila* (Goldman et al. 2005) as well as on *H. virescens* and *H. subflexa* (Baker et al. 2004) provide evidence for dual OR expression on a single ORN. In *O. nubilalis*, expression of two different ORs on the ORN involved in behavioral antagonism would imply an adaptive significance. This possibility would have been better supported if our analysis of variance for the paired stimulation experiments for these two compounds had shown a significant effect of cross- *versus* self-adaptation, or an interaction between cross- *versus* self-adaptation and the compound puffed. Thus, it appears that the neuronal adaptation caused by either of these compounds has an identical effect on subsequent stimulations, a pattern that would occur if both compounds bind to the same OR. However, such a pattern remains possible for a two-ORN model if recovery from adaptation is caused by factors other than OR saturation. Whether one or two ORs are involved in the behavioral antagonism observed in response to Z11-16:Ald and Z9-14:Ac, the result would be increased conspecific pheromone blend discrimination and, therefore, less cross-attraction in an environment occupied by other species that share a subset of the same compounds in their pheromone blends.

Acknowledgment We thank Kathy Poole for her help in maintaining the ECB colonies. The project was funded by NSF IBN #034340.

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J Chem Ecol (2007) 33:923–933
DOI 10.1007/s10886-007-9266-5

Contact Sex Pheromone Components of the Cowpea Weevil, *Callosobruchus maculatus*

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Received: 11 September 2006 / Accepted: 15 February 2007 /
Published online: 30 March 2007
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Abstract The cowpea weevil, *Callosobruchus maculatus*, is a major pest of stored pulses. Females of this species produce a contact sex pheromone that elicits copulation behavior in males. Pheromone was extracted from filter-paper shelters taken from cages that housed females. Crude ether extract stimulated copulation in male *C. maculatus*. Initial fractionation showed behavioral activity in acidic and neutral fractions. Furthermore, bioassay-guided fractionation and gas chromatography-mass spectroscopy (GC-MS) analysis of active fractions revealed that the active components of the acidic fraction were 2,6-dimethyloctane-1,8-dioic acid and nonanedioic acid. These components along with the hydrocarbon fraction, a mixture of C_{27}–C_{35} straight chain and methyl branched hydrocarbons, had a synergistic effect on the behavior of males. Glass dummies treated with an authentic pheromone blend induced copulation behavior in males. The potential roles of the contact sex pheromone of *C. maculatus* are discussed.

Keywords *Callosobruchus maculatus* · 2,6-Dimethyloctane-1,8-dioic acid · Nonanedioic acid · Hydrocarbon · Female contact sex pheromone · Cowpea weevil · Coleoptera · Bruchidae

Introduction

Sex pheromones are chemical signals required for the mating behavior of many insect species and are categorized according to their function in the mating sequence. For

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example, sex attractant pheromone is released by one sex and attracts the other sex from a distance. Contact sex pheromone is also produced by one sex, but it works at close range to elicit courtship and/or copulation behavior in the other sex. Generally, sex attractant pheromones are highly volatile, olfactory-perceived stimuli, whereas contact sex pheromones have low volatility and act as contact or gustatory stimuli (Birch 1974). Although many sex attractant pheromones have been identified, mainly from pest species (http://www.pherobase.org), fewer contact sex pheromones have been identified, probably due to their lesser potential for pest control applications. Both attractant and contact sex pheromones can, however, be important for mating success, and in some species, both types of pheromones are used sequentially (e.g., *Blattella germanica*; Nojima et al. 2005).

Callosobruchus spp. are major pests of stored pulses, cowpeas, pigeonpeas, and other legumes (Rees 2004). Females of *Callosobruchus chinensis* produce both sex attractant and contact sex pheromones that are distinct from each other (Tanaka et al. 1981). The sex attractant pheromone of *C. chinensis* has been identified recently as a mixture of C_{16} aldehydes (Shimomura, personal communication). The contact sex pheromone of *C. chinensis* has been characterized as a mixture of a monoterpene dicarboxylic acid and C_{25}–C_{35} straight chain and methyl branched hydrocarbons (Tanaka et al., 1981). Glass rod dummies treated with a synthetic female contact pheromone blend elicited copulation behavior from males (Tanaka et al. 1982). Dummies equipped with an artificial genital opening stimulated genital insertion and ejaculation (Honda and Ohsawa 1990).

Callosobruchus maculatus is closely related to *C. chinensis* and is also an important pest of stored pulses (Rees 2004). The presence of a sex attractant pheromone in this species was demonstrated by Rup and Sharma (1978), and the pheromone was later characterized as a mixture of short-chain fatty acids (Phillips et al. 1996). Various biological and behavioral aspects of the sex attractant pheromone have been studied, including pheromone production (Qi and Burkholder 1982; Lextrait et al. 1995; Shu et al. 1996), male response patterns to the pheromone (Lextrait et al. 1994), pheromone gland morphology (Ramaswamy et al. 1995), and the role of the attractant pheromone for discrimination of conspecific males and related species (Phillips et al. 1996; Mbata et al. 2000). However, the contact sex pheromone of *C. maculatus* has not been chemically investigated. Here, we characterize the *C. maculatus* female contact sex pheromone to understand the pheromonal communication system of this species better.

Methods and Materials

Insects A laboratory-maintained colony was used in the experiments. Insects were reared on *Vigna angularis* (variety Dainagon from Hokkaido, Japan) in a dark incubator at 27°C and 60% relative humidity. Newly emerged adults were sexed and maintained under the same conditions as above. Females were kept in a group in glass containers (90 mm ID× 60 mm height) with corrugated filter paper shelters (90 mm ID, Advantec No. 2).

Pheromone Collection Virgin females were kept in containers for 3–4 wk. Filter-paper shelters were collected and Soxhlet-extracted with ether for 20 hr. The insides of the glass containers were rinsed with ether, and the ether combined with the extracts. The solvent was evaporated under reduced pressure. Extracts were accumulated from a total of ca 3,000 females and kept in a refrigerator until use.

Bioassays Newly emerged males were held singly in capped glass vials (1.5 cm i.d. and 3.0 mm in height) and kept for 1 d in the incubator under the same conditions as for

rearing. Males were taken from the incubator and acclimated to the light conditions 1 hr before the behavioral tests. A glass rod (2 mm OD and 150 mm in length) was used as a female dummy. The tip of the rod was rounded by the flame of a gas burner and abraded with # 1000 abrasive paper. Four microliters of ethereal extract were applied to the tip of the rod with a micro syringe. After the solvent evaporated, the female dummy was introduced into a vial, and a male's antennae gently rubbed with the dummy for 2 min. In response to the contact sex pheromone, males showed characteristic copulation behaviors: drumming the dummy with the antennae, holding it with the forelegs, and extruding the genital organ toward the dummy. The number of males attempting genital connection with the dummy was recorded. The dummy was changed every ten males. For semiqualitative assays, each sample was tested using 20 or 30 males, and the results were analyzed by Fisher's exact probability followed by the sequential Bonferroni test (Rice 1989). For quantitative assays, the relation between a series of dosages of a treatment and copulation-release activity was subjected to probit analysis, and the median effective dosage (ED_{50}) estimated. A parallel-line assay was conducted on a set of preparations to estimate a common regression formula by using a computer program by Sakuma (1998). The validity of the results of the parallel-line assay was examined by χ^2 analysis of heterogeneity of the observed responses deviating from the expected. If heterogeneity was significantly large ($P<0.05$), a variance ratio test [analysis of variance (ANOVA)] between parallelism and heterogeneity was used to verify the results. Ten to 80 males were tested to each dosage of a treatment, and a total of 140 to 300 males were used for a preparation to estimate the ED_{50} value of a sample. Sample dose was adjusted in terms of female equivalents (f.e.).

Purification of Pheromone Components Crude pheromone extract (ca 3,000 f.e.) was dissolved in 50 ml ether, and the solution was fractionated into neutral, acidic, and basic fractions with 5% aq. NaOH and 5% aq. HCl (Fig. 1). Acidic compounds were redissolved in ether, and the solution concentrated to dryness with Celite 545 (100 mg) in a rotary evaporator. The Celite was suspended in methanol–water–trifluoroacetic acid in a ratio of 20:80:1 (*v*/*v*/*v*), and the suspended mixture applied to the top of a prewetted ODS column [Isolute C18 (EC) 500 mg, International sorbent technology Ltd. Mid Glamorgan, UK]. Acidic components absorbed on the Celite were eluted successively with 10 ml of methanol–water–trifluoroacetic acid in a ratio of 20:80:1 (*v*/*v*/*v*) and 5 ml each of 40:60:1, 60:40:1, 80:20:1, and 100:0:0 (Fig. 1). The active fraction was further fractionated by column chromatography on 100 mg of silica gel (Wakogel C-300, Wako Pure Chemical Industries Ltd. Osaka, Japan). The column was eluted with a sequence of 1 ml aliquots of hexane–ether–trifluoroacetic acid in ratios of 80:20:0.2 (*v*/*v*/*v*), 70:30:0.2, 60:40:0.2, 50:50:0.2, and 35:65:0.2 and methanol (Fig. 1). Acidic compounds in the active fraction were derivatized to methyl esters (see below) and subjected to gas chromatography-mass spectroscopy (GC-MS) analysis. The neutral fraction was chromatographed on 5 g of silica gel (Wakogel C-200, Wako Pure Chemical Industries Ltd. Osaka, Japan) and eluted successively with 50 ml each of hexane–ether in a ratio of 100:0 (*v*/*v*), 95:5, 90:10, 80:20, 50:50, and 0:100 (Fig. 2). The active fraction was subjected to GC-MS analysis.

Methyl Esterification of Acidic Components An aliquot of the acidic fraction was placed in a small conical vial, and mixed with 5 μl methanol and 10 μl of 0.1% (trimethylsilyl) diazomethane in hexane solution (Tokyo Kasei, Co. Ltd. Tokyo, Japan). The mixture was left at room temperature for 30 min with occasional stirring, and subjected to analysis by GC-MS.

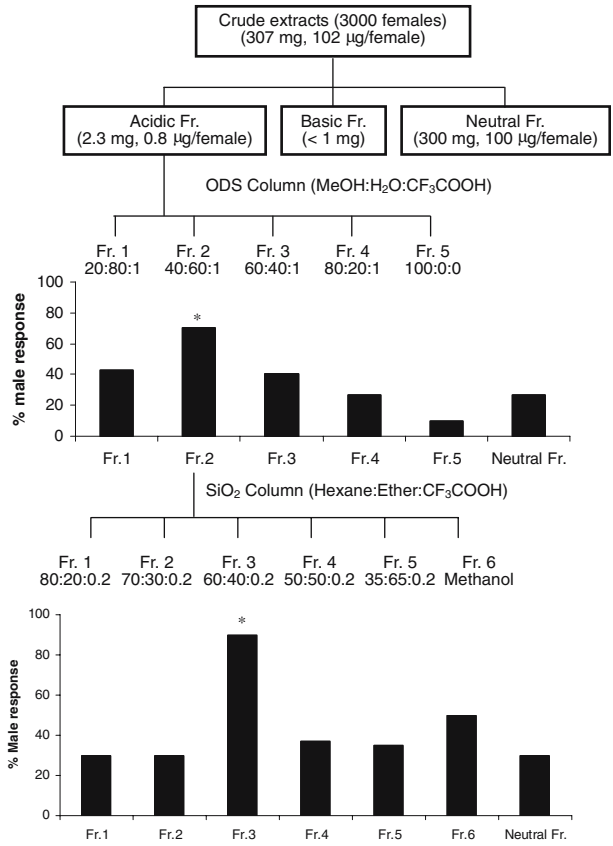


Fig. 1 Fractionation procedure for crude pheromone extract of female *Callosobruchus maculatus* and bioassay-response data for each fraction. Fractions from the reverse-phase separation were tested at 0.05 female equivalents, and fractions from the normal-phase separation at 0.1 female equivalents. All fractions were augmented with the neutral fraction at the same concentration. *Asterisks* indicate statistical differences between the neutral fraction and the test fraction (Fisher's exact probability test followed by the sequential Bonferroni test, $P<0.05$)

Identification of Hydrocarbon Components Hydrocarbon components in the hexane fraction were tentatively identified by comparison with published GC profiles, Kovats retention indices, and mass fragmentation patterns (Baker and Nelson 1981; Howard 2001).

Instruments GC-MS analysis was performed on a Shimadzu GC-17A equipped with 30 m×0.25 mm ID, 0.25 µm film thickness DB-1 or DB-23 columns (J&W scientific, Folsom, CA, USA) in splitless mode, coupled to a Shimadzu QP-5000 quadrupole MS in EI scan mode (electron ionization at 70 eV). Helium was used as the carrier gas at a head pressure of 100 kPa (flow rate, 1.3 ml/min). For analysis of methyl ester derivatives, oven temperature was initially 100°C for 2 min, then increased by 4°C/min to 220°C and held at this temperature for 2 min. Injector and interface temperatures were set at 250°C for methyl ester analysis on both nonpolar and polar columns. For hydrocarbon analysis on the DB-1 column, two temperature programs were used for measurement of Kovats retention indices: (a) initial temperature of 100°C for 2 min, increased by 16°C/min to 330°C and held for 5 min or (b) 200 to 350°C at 8°C/min. Injector and detector temperatures were set at 350 and 340°C, respectively.

Chemicals 2,6-Dimethyloctane-1,8-dioic acid was prepared by hydrogenation of dimethyl ester of (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid (>95% purity), available from a previous study (Tanaka et al. 1982). Hydrogenation was performed by treating the dimethyl ester in

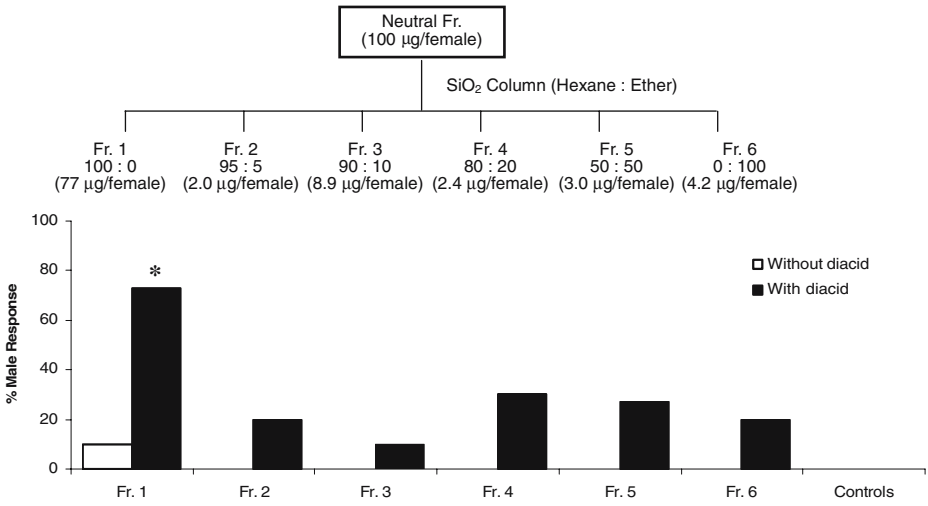


Fig. 2 Fractionation procedure for the neutral fraction of female *Callosobruchus maculatus* extracts, and bioassay-response data for each fraction at 0.05 female equivalents. Each fraction was tested with ("with diacid") and without ("without diacid") 10 ng of synthetic 2,6-dimethyloctane-1,8-dioic acid. Female dummies treated with solvent or the diacid are shown as "without diacid" and "with diacid" controls, respectively. Samples without acid were compared with the "without diacid" control, and samples with acid with the "with diacid" control. *Asterisks* indicate statistical differences between the fractions and controls (Fisher's exact probability test followed by the sequential Bonferroni test, $P<0.05$)

ether with 10% Pd on activated charcoal catalyst and passing hydrogen gas through the solution. Dimethyl ester of 2,6-dimethyloctane-1,8-dioic acid was obtained as a mixture of stereoisomers. The mixture was hydrolyzed with 20% $NaOH_{(aq)}$, and the dicarboxylic acids extracted with ether after acidification with concentrated HCl. The purity of the dicarboxylic acid, as a mixture of stereoisomers, was >95% by GC analysis after methyl ester derivatization. Nonanedioic acid, hydrocarbons, and liquid paraffin were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Results

Preliminary Purification of the Crude Extracts Female contact sex pheromone was extracted from the filter papers taken from female containers. The yield of crude extract from 3,000 females was 307 mg. The extract elicited strong copulation behavior from males; all males tested attempted to copulate with female dummies treated with 0.05 f.e. crude extract (Table 1). The ED_{50} of crude extract was estimated at 0.0064 f.e. (Table 2). When the crude extracts were fractionated into acidic, basic, and neutral fractions (Fig. 1), only the neutral fraction elicited copulation activity, albeit weakly, with 27% of males responding to the dummies with 0.05 f.e. (Table 1). The original level of activity was restored when the acidic and neutral fractions were combined (Table 1).

Purification and Identification of Acidic Pheromone Components The acidic fraction was further fractionated by reverse-phase liquid chromatography (LC). The resultant fractions were mixed with the neutral fraction and subjected to behavioral assay. Activity was found

Table 1 Copulation responses of male *C. muculatus* to crude pheromone extract, and acidic, basic, and neutral fractions of the extract

Sample[a]	Responses (%)[b,c]
Crude extract	100
Acidic fraction	0
Basic fraction	0
Neutral fraction	27
Acidic+neutral fraction	100*
Solvent control	0

[a] Tested at 0.05 female equivalents.

[b] Thirty virgin males were used for each sample.

[c] Asterisk indicates sample response not significantly different from the crude extract (Fisher's exact probability test followed by the sequential Bonferroni test, $P>0.05$).

in the fraction that eluted with 40:60:1 (*v*/*v*/*v*) methanol–water–trifluoroacetic acid (Fig. 1). This fraction was fractionated by normal-phase LC, with activity found in the 60:40:0.2 (hexane–ether–trifluoroacetic acid) fraction (Fig. 1). Acidic components in this active fraction were converted to methyl esters and subjected to GC-MS analysis.

Two major components were found in the active fraction, compounds **1** and **2** (Fig. 3). The mass spectrum of compound **1** showed a base peak at *m/z* 55 and characteristic fragments at *m/z* 74 (22) and 88 (75), suggesting McLafferty rearrangements of two methyl ester moieties; $–CH_2–CO–OCH_3$ and $–CH(CH_3)–CO–OCH_3$, respectively. The highest observable ion of compound **1** was *m/z* 199, probably resulting from loss of a methoxy group (M-31) from a molecule with molecular weight of *m/z* 230. Thus, compound **1** was assigned as a dicarboxylic acid with a methyl branch at the α-position. A monoterpene dicarboxylic acid, (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid, has been identified from a related species, *C. chinensis* (Tanaka et al. 1981, 1982). This compound has a methyl branch at the α-position, and its dimethyl ester has a molecular mass of *m/z* 228.

Table 2 Effect of synthetic chemicals on copulation release activity of males of *C. maculatus*

Groups $Y=A+BX$ (χ^2 test; ANOVA)[a] Sample	*A*	ED_{50} (fiducial limits)[b] (female equivalents)
1. $Y=A+0.7455X$ ($P<0.05$; ANOVA, $P>0.05$)		
2,6-Dimethyloctane-1,8-dioic acid (1)	−1.383	72 (21–300)
Nonanedioic acid (2)	−3.267	24,000 (6,800–150,000)
Paraffin+(1)+(2)	0.119	0.69 (0.22–2.7)
2. $Y=A+4.277X$ ($P>0.05$)		
Crude extract	9.369	0.0064 (0.0056–0.0073)
Neutral Fr.+(1)+(2)	5.863	0.043 (0.037–0.049)
Hydrocarbon Fr.+(1)+(2)	–	ND[c] (–)

[a] Regression formula by probit analysis. (A) Section for each sample. (B) Common regression coefficient. Significance was examined by a χ^2 test of heterogeneity. If it was large ($P<0.05$), parallelism was examined by ANOVA between parallelism and heterogeneity.

[b] Median effective dosage with 95% fiducial limits in parentheses. Heterogeneity was large ($P<0.05$) in group **1**, so a *t*-deviate was used for *t*, while heterogeneity was small ($P>0.05$) in group **2**, so a normal deviate was used for *t*.

[c] ND The median effective dosage was not determined due to low activity.

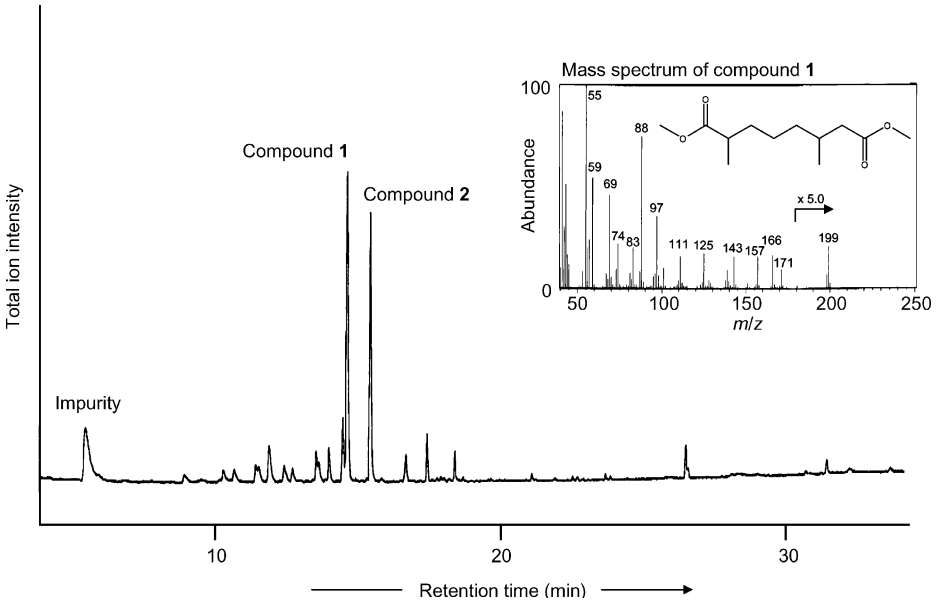


Fig. 3 Total ion chromatogram of the active fraction resulting from normal-phase liquid chromatography of the original acidic fraction. The active fraction was methyl esterified before injection into the GC-MS. *Inset* is mass spectrum and assigned structure of compound **1**

Comparison of the mass spectrum (Tanaka et al. 1981) of the methyl ester of hydrogenated (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid (i.e., 2,6-dimethyloctane-1,8-dioic acid dimethyl ester; EI at 20 EV) with that of compound **1** (EI at 70 eV) showed similarities, although with some differences in the relative abundance of fragment ions. The differences are likely due to the different electron voltages used in the two studies. Therefore, we assigned compound **1** as 2,6-dimethyloctane-1,8-dioic acid dimethyl ester. The mass spectrum of compound **2** matched nonanedioic acid dimethyl ester in a library search.

The identities of compounds **1** and **2** were confirmed by comparing GC retention times on two different columns (DB-1 and DB-23) and EI-MS spectra with those of authentic standards. The amounts of compounds **1** and **2** were quantified as 3.0 and 2.5 ng/female in the extracts, respectively.

Purification and Chemical Analysis of Neutral Pheromone Components The neutral fraction was subjected to normal phase LC. Each fraction was tested in the behavioral assay, alone or with synthetic 2,6-dimethyloctane-1,8-dioic acid. The hexane fraction alone elicited slight copulatory activity. However, this activity was enhanced significantly by adding 2,6-dimethyloctane-1,8-dioic acid (Fig. 2). GC-MS analysis of this fraction revealed a number of hydrocarbons, tentatively identified as a mixture of C_{27}–C_{35} hydrocarbons, *n*-alkanes, and a homologous series of mono- and di-methyl branched alkanes (Fig. 4), by comparing retention indices and MS fragmentation patterns with those in the literature (Baker and Nelson 1981; Howard 2001). The total amount of hydrocarbons in the hexane fraction was 77 μg/female (Fig. 2).

Copulation Release Activity The copulation-release activities of 2,6-dimethyloctane-1,8-dioic acid and nonanedioic acid were investigated with authentic standards. The di-

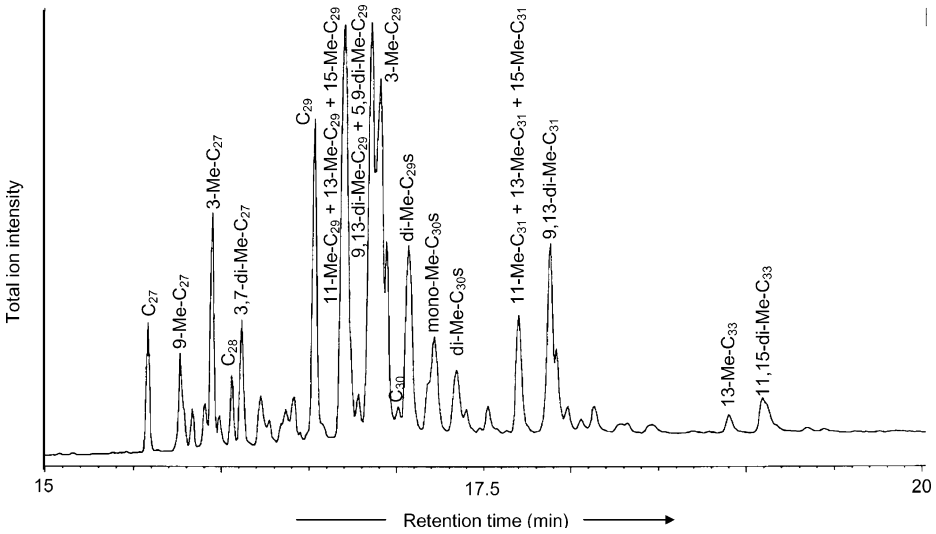


Fig. 4 Total ion chromatogram of the hydrocarbon fraction of female contact pheromone of *Callosobruchus maculatus*

carboxylic acids alone elicited full copulation behavior from the males. All tested males attempted to copulate with female dummies treated with 10 μg of 2,6-dimethyloctane-1,8-dioic acid, while 43% of males responded to 100 μg of nonanedioic acid. The ED_{50} of the dicarboxylic acids was estimated by probit analysis of the relationship between dose and response. A parallel-line analysis was conducted to compare ED_{50} values between samples. This failed ($P<0.05$) because the regression coefficient provided by independent probit analyses of each sample varied widely (0.47–5.0). Consequently, the samples were grouped as shown in Table 2 according to the regression coefficient in the independent analyses. A parallel-line assay was conducted within each group again, with validity of parallelism confirmed in each group. The ED_{50} values of 2,6-dimethyloctane-1,8-dioic acid and nonanedioic acid (group 1) were estimated at 0.22 μg (ca 72 f.e.) and 60 μg (ca 24,000 f.e.), respectively (Table 2). The activity of 2,6-dimethyloctane-1,8-dioic acid was significantly higher than that of nonanedioic acid both on a weight and a f.e. basis. It is important to note, however, that f.e. calculations are based on filter-paper extracts, not females.

The copulation release activity of the hydrocarbons was investigated by using commercially available octacosane, nonacosane, and triacontane. Nonacosane is a major component in the hexane fraction, whereas octacosane, and triacontane are minor components (Fig. 4). Hexadecane, octadecane, and liquid paraffin (a mixture of liquid hydrocarbons from petroleum) were also tested. None of the hydrocarbons tested alone at 0.05 f.e. (3.6 μg/dose) or at a higher dosage of >1 mg (data not shown) elicited copulation activity (Fig. 5). With the addition of 10 ng of authentic 2,6-dimethyloctane-1,8-dioic acid, the reference hydrocarbons, octadecane, and liquid paraffin showed significant activity ($P<0.05$, Fig. 5) relative to the control. Interestingly, in combination with the diacid, liquid paraffin, or octadecane elicited the same level of copulatory behavior as did the same amounts of the natural hydrocarbon fraction or the neutral fraction in combination with the diacid ($P>0.05$).

Copulation release activity of artificial and semi-artificial pheromone blends was investigated by comparison of their ED_{50} values. The ED_{50} of a mixture of synthetic 2,6-dimethyloctane-1,8-dioic acid, nonanedioic acid, and neutral fraction, was estimated at

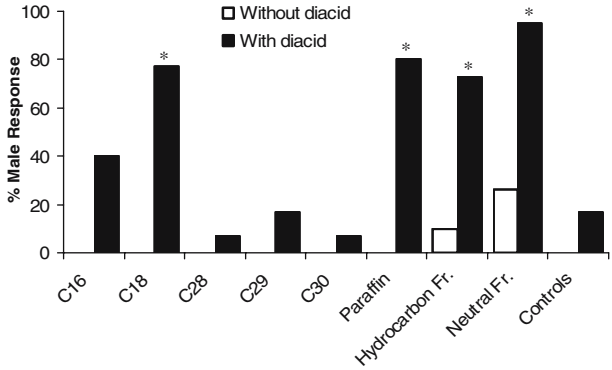


Fig. 5 Bioassay data testing various hydrocarbons and fractions added to 10 ng of the acidic pheromone component ("with diacid") or solvent ("without diacid"). The activities of samples without the diacid were compared with the "without acid" control, while samples augmented with the acid were compared with the "with diacid" control. *Asterisks* indicate statistical differences between treatment and control tests (Fisher's exact probability test, followed by the sequential
Bonferroni test, $P<0.05$)

0.043 f.e. (group 2), whereas a blend of authentic dicarboxylic acids and the natural hydrocarbon fraction showed less activity; its ED_{50} was not determined due to low activity. On the other hand, the ED_{50} of a blend of authentic dicarboxylic acids and liquid paraffin (group 1) was estimated to be 0.69 f.e. (Table 2).

Discussion

The female contact sex pheromone of *C. maculatus* was characterized as a mixture of dicarboxylic acids and hydrocarbons. Behavioral assays with authentic standards revealed that high doses of the acids alone induced full copulation behavior in males, with the activity significantly enhanced by addition of hydrocarbons. Hydrocarbons alone were marginally active.

In this study, the female contact sex pheromone of *C. maculatus* was collected from filter papers taken from containers with virgin females because in a preliminary experiment, body extracts did not consistently elicit copulation activity from males. After contact with dummies treated with female body extracts, most males groomed their antennae and did not attempt to copulate, possibly due to the large quantities of chemicals and the sticky nature of the extracts. In contrast, the extracts of filter papers from the female containers elicited consistent copulation activity in males. The female contact sex pheromone is probably transferred to the filter paper when females rub against it. It seems likely that the active chemicals are cuticular in origin as ca 75% of the mass of crude extracts was hydrocarbons with a GC profile almost identical to that reported previously (Baker and Nelson 1981).

Insect cuticular hydrocarbons play important roles in chemical communication, as well as having ecological and physiological roles (Howard 1993). Many insect species use cuticular hydrocarbons as mate recognition signals with the composition, or specific structures, of the hydrocarbon components apparently important (Howard 1993). In *C. maculatus*, female cuticular hydrocarbons are important in pheromonal communication. However, the specificity of these cuticular hydrocarbons may not be great as non-native hydrocarbons such as hexadecane, octadecane, and liquid paraffin elicited similar or even

greater behavioral activity than the natural hydrocarbon fraction. Interestingly, octacosane, nonacosane, and triacontane, which are solid at room temperature (m.p. 57–62, 63–66, and 64–67°C, respectively), did not increase activity when added to the acids, whereas hexadecane, octadecane, and liquid paraffin, which are liquids at room temperature (m.p. of C_{16} and C_{18} are 18 and 26–29°C, respectively), did. Although our experiments were not designed to investigate the relationship between activity and physical properties of the hydrocarbons, perhaps, in this insect, the physical nature of cuticular hydrocarbons is more important than their specific structure or composition.

A female contact sex pheromone of *Callosobruchus* spp. was first characterized for *C. chinensis*. The pheromonal mixture of (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid and C_{25}–C_{35} straight chain and methyl branched hydrocarbons (Tanaka et al. 1981, 1982) is chemically similar to that which we found for *C. maculatus*. In fact, high proportions of males, of both *C. chinensis* and *C. maculatus*, exhibited copulation behavior to females of the other species in laboratory bioassays (Noijima et al. unpublished observation). Although the respective contact sex pheromones of these two species appear to lack species specificity, their different sex attractant pheromones likely preclude males from finding and encountering females of the other species.

In the artificial pheromone blend experiments, a synthetic mixture of 2,6-dimethyloctane-1,8-dioic acid, nonanedioic acid, and liquid paraffin elicited copulation activity in males with an ED_{50} value much higher than that of crude pheromone extract (ED_{50}, 0.0064 f.e.), suggesting that additional active chemicals may be present in the filter paper extract. The yellow mealworm, *Tenebrio molitor* L. (Coleoptera, Tenebrionidae) has a similar pheromone system, with a female sex attractant and nonvolatile contact sex pheromone (Tanaka et al. 1986). The attractant sex pheromone, in combination with the contact sex pheromone, enhances copulation activity as much as 25 times over the contact pheromone alone (Tanaka et al. 1986). A female sex attractant pheromone for *C. maculatus* was first demonstrated with extracts of filter paper from a vial containing a female (Qi and Burkholder 1982), similar to that used in our study. Therefore, the greater amount of copulation activity elicited by crude extracts, compared to fractions or synthetic components, may have been due to the presence of sex attractant pheromone in the crude extracts. In addition, there may be other components in the contact pheromone, as the neutral fraction alone elicited more behavioral activity than its hydrocarbon subfraction.

In insect pheromone communication, the absolute configuration of pheromone components is often important for behavioral activity (Mori 1997, 2000). The female contact sex pheromone component of *C. maculatus*, 2,6-dimethyloctane-1,8-dioic acid, has two chiral centers, and thus, four stereoisomers are possible. Recently, all four enantiomers of this chemical were synthesized, and it was shown that the natural pheromone of *C. maculatus* is a mixture of all four (Nakai et al. 2005; Yajima et al. 2006). Moreover, each enantiomer elicits copulation behavior in males at similar doses, without antagonism to the others. This is similar to the situation in the related species, *C. chinensis*. In this species, the contact sex pheromone component, (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid, has one chiral center (Tanaka et al. 1981), and the two enantiomers elicit similar behavioral activity (Mori et al. 1983). Further study will be carried out to clarify the structure–activity relationships of chemicals in the contact pheromone of *C. maculatus*.

Acknowledgements The authors wish to express their gratitude to Drs. Kenji Masuyama and Kaoru Tanaka for their valuable comments and help. We also express our gratitude to Dr. Coby Schal and Elsa Youngsteadt for reviewing the manuscript.

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J Chem Ecol (2007) 33:935–945
DOI 10.1007/s10886-007-9269-2

Queen Sex Pheromone of the Slave-making Ant, *Polyergus breviceps*

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Received: 30 June 2006 / Accepted: 21 February 2007 /
Published online: 29 March 2007
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Abstract Workers of the slave-making ant, *Polyergus breviceps*, raid nests of *Formica* ants and return with *Formica* pupae that mature into worker ants in the slave-makers' colony. These *Formica* workers then tend the *Polyergus* brood, workers, and reproductives. During raids in the mating season, winged virgin *Polyergus* queens accompany the workers in the raiding columns. During the raid, the virgin queens release a pheromone that attracts males that quickly mate with the queens. We report the identification, synthesis, and bioassay of the sex attractant pheromone of the queens as an approximately 1:6 ratio of (*R*)-3-ethyl-4-methylpentan-1-ol and methyl 6-methylsalicylate. The ants produce exclusively the (*R*)-enantiomer of the alcohol, and the (*S*)-enantiomer has no biological activity, neither inhibiting nor increasing attraction to blends of methyl 6-methylsalicylate with the (*R*)-enantiomer.

Keywords *Polyergus breviceps* · Queen sex pheromone · Methyl 6-methylsalicylate · (*R*)-3-ethyl-4-methylpentan-1-ol · Ant · Formicidae · Hymenoptera

Introduction

Ant species in the genus *Polyergus* Latreille (Hymenoptera: Formicidae) are obligate social parasites that have lost the ability to forage for food, rear brood, or care for the queen and the nest. They are entirely dependent upon workers of slave ant species to carry out the tasks that enable a colony to survive (Topoff 1999). Because the slave species do not breed within the *Polyergus* nest, slave ants are captured as pupae during periodic raids conducted by *Polyergus* workers against related ants in the genus *Formica* Linneaus. The captured

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pupae are carried back to the *Polyergus* nest, and as the adults of the slave species emerge they are integrated into the nest, where they assume all the typical foraging and "housekeeping" functions that workers normally carry out.

In the deserts of southwestern Arizona, USA, closely corresponding with summer rains in early July, *Polyergus breviceps* Emery males and virgin queens start to congregate at nest entrances in mid-afternoon. Males are usually seen first and may start flying from the nest at this time. If the *Polyergus* workers initiate a slave raid against a *Formica* colony (usually between 1500 and 1800 hours), winged virgin *Polyergus* queens accompany the raiding workers. During the course of the raid, the virgin queens release a pheromone that rapidly attracts males. Copulation lasts for about 15 sec to several min, after which the mated queen sheds her wings. Then, the newly mated *Polyergus* queens independently search for, invade, and take over a colony of *F. gnava* Buckley (the common slave species at this location), first killing the resident *Formica* queen (Topoff et al. 1985; Topoff and Zimmerli 1993; Johnson et al. 2002). It is not known whether a queen invades a *Formica* nest immediately after a slave raid while the workers are still disorganized, or on subsequent days. The *Polyergus* queen acquires the cuticular hydrocarbon profile of the host queen that she has killed and is subsequently tended by the host workers (Johnson et al. 2001). The *Formica* workers then rear the *Polyergus* queen's brood as their own, giving rise to a mixed colony of workers of the two species.

A preliminary study of sex pheromone-mediated behaviors of *P. breviceps* was initiated after observing that *Polyergus* males were attracted to a vial in which a virgin queen had been inadvertently squashed (Topoff and Greenberg 1988). When the various body parts of a fresh queen were tested, only air flowing over the squashed head elicited a response from males, causing them to rapidly run in circles and to attempt to mate with each other. The same response was observed when only the mandibular glands were tested, whereas a head with the glands removed did not elicit any response.

Methyl 6-methylsalicylate had been reported as the major component of the mandibular glands of *Polyergus rufescens*, the European congener of *P. breviceps* (Castracani et al. 2003; Grasso et al. 2003), and in field trials it was reported to attract male ants (Castracani et al. 2005). However, these authors suggested that a second compound also may be involved in the queen pheromone, possibly as a component of the secretion of the newly described intramandibular gland (Grasso et al. 2004).

In a recent rapid communication (Greenberg et al. 2004), we reported that the queen-produced pheromone of *P. breviceps* consisted of an approximately 3:1 blend of methyl 6-methylsalicylate and racemic 3-ethyl-4-methylpentan-1-ol. We also provided a brief description of the field trials and chemical analyses that resulted in the identification. Here, we report the syntheses of the enantiomers of the chiral component, 3-ethyl-4-methylpentan-1-ol. We further report the determination of the absolute configuration of the 3-ethyl-4-methylpentan-1-ol produced by the queen ants, and comparative bioassays of pheromone blends composed of the racemate or its individual enantiomers, in combination with the second component of the pheromone, methyl 6-methylsalicylate.

Methods and Materials

Insect Samples

Winged (virgin) queen *P. breviceps* were collected at the Southwestern Research Station of the American Museum of Natural History in Portal, Arizona. Queens were collected from raiding columns by aspiration, immediately killed by freezing, and cut into head, thorax,

and abdomen. Frozen body parts in 1.5-ml screw cap glass vials were shipped on dry ice to University of California Riverside for analysis. Voucher specimens of *P. breviceps* and *Formica gnava* are archived at the UC Riverside Entomology Museum (UCRC ENT 131329–131343 and 131328, respectively).

Preparation and Analysis of Extracts

Solid phase microextraction (SPME) collections of volatiles released from virgin queen heads were made by crushing heads in their vials with a glass rod, sealing the vial with aluminum foil, pushing the SPME needle through the foil, and exposing the SPME fiber (100 μm polydimethylsiloxane coating; Supelco, Bellefonte, PA, USA) in the headspace for 30 min. The loaded fibers then were desorbed in the injection port of a coupled gas chromatograph-mass spectrometer (GC-MS) for 30 sec at 250°C, in splitless injection mode. GC-MS analyses of SPME extracts were carried out with a Hewlett-Packard 6890 GC (H-P; Avondale, PA, USA) with liquid CO_2 cryogenic cooling, equipped with a 30 m×0.25 mm HP-5 MS column, interfaced to an HP 5973 mass selective detector (electron impact ionization, 70 eV), using a GC temperature program of 0° for 1 min, then 10° per minute to 250°C.

To determine the absolute configuration of the insect-produced 3-ethyl-4-methylpentanol, SPME-collected volatiles from squashed heads of virgin queens were analyzed on a Cyclodex-B column (J&W Scientific, 30 m×0.25 mm id, 0.25 film; Folsom, CA, USA) with a head pressure of 140 kPa and a temperature program of 50°C for 1 min, then 3° per minute to 240°C. The retention times of synthetic standards of the (3*R*)- and (3*S*)-enantiomers under these conditions were 19.64 and 19.76 min, respectively. The same conditions were used to determine the enantiomeric purity of synthetic compounds.

Syntheses of Pheromone Components

All reactions were carried out at room temperature in oven-dried glassware unless stated otherwise. Methyl 6-methylsalicylate (Seidel et al. 1990) and racemic 3-ethyl-4-methylpentanol standards (Bühring et al. 1976) were synthesized as previously described. The first synthesis of enantiomerically enriched 3-ethyl-4-methylpentanol enantiomers was carried out as described by Nicotra et al. (1986), providing the (*R*)-and (*S*)-enantiomers in 89.0 and 86.4% enantiomeric excess (ee), respectively. This material was used in bioassays in 2004. A second synthesis subsequently was carried out to provide material of higher ee, starting from commercially available (*R*)-(+)-limonene **2**, as follows (Fig. 1).

(2RS,5S)-5-Ethyl-6-methylheptan-2-ol (5) Commercially available (*R*)-(+)-limonene **2** was hydrogenated to (*R*)-(+)-dihydrolimonene **3** as described by White et al. (1981). Alkene **3** was then transformed to (3*R*,6*RS*)-3-(1-methylethyl)heptane-1,6-diol 4 by the method of Nicotra et al. (1986).

Under an atmosphere of dry nitrogen, (3*R*,6*RS*)-3-(1-methylethyl)heptane-1,6-diol **4** (1.29 g, 7.40 mmol) was dissolved in 8 ml dry pyridine and cooled to 0°C. *p*-Toluenesulfonyl chloride (1.72 g, 9.02 mmol) dissolved in 12 ml dry pyridine was added to the stirred solution over 2 hr, and stirring was continued for 24 hr at room temperature. The mixture then was diluted with 40 ml diethyl ether. This mixture and 120 ml dilute hydrochloric acid (3% solution in brine) were added separately to 150 ml ice-cold brine so that the solution was kept at pH 6. After separation of the layers, the aqueous layer was

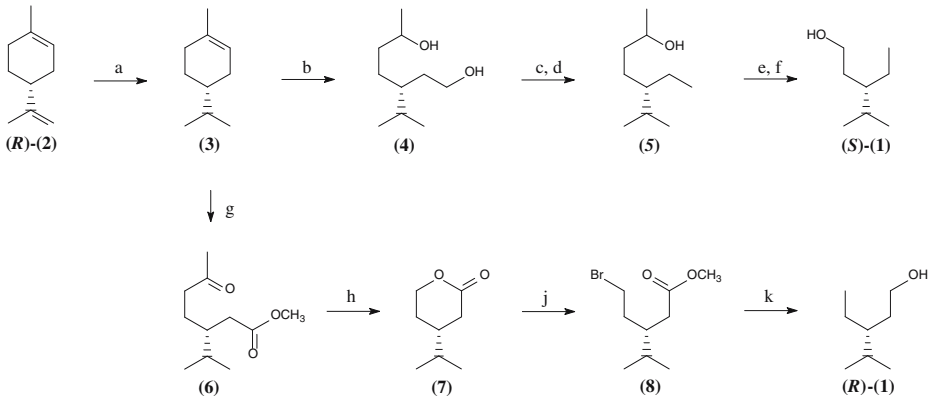


Fig. 1 Syntheses of (*S*)-(-)- and (*R*)-(+)-3-ethyl-4-methylpentan-1-ol. (a) PtO_2, H_2, MeOH; (b) (1) O_3, CH_2Cl_2/MeOH, −70°C; (2) $NaBH_4$ −70°C; (c) *p*-TsCl, pyridine, 0°C; (d) $LiAlH_4/Et_2O$, −30°C; (e) PDC, CH_2Cl_2; (f) H_2O_2, aq. NaOH/MeOH; (g) (1) O_3, CH_2Cl_2/MeOH, −70°C, (2) *p*-TsOH, −70°C to room temp, dimethylsulfide; (h) H_2O_2, aq. NaOH/MeOH; (j) HBr, MeOH, −30°C; (k) $LiAlH_4/Et_2O$, −20°C

extracted five times with 20 ml diethyl ether. The combined organic extracts were washed with 15-ml portions of saturated $NaHCO_3$, brine, aqueous cupric sulfate, and brine. After drying over anhydrous (anhyd.) $MgSO_4$, the solvent was removed *in vacuo* to yield 1.90 g of crude (3*R*,6*RS*)-3-(1-methylethyl)-1-*p*-toluenesulfonyloxyheptan-6-ol, which was used without further purification.

Under an atmosphere of dry nitrogen, $LiAlH_4$ (530 mg, 14 mmol) was suspended in 30 ml dry diethyl ether and cooled to −30°C. Crude (3*R*,6*RS*)-3-(1-methylethyl)-1-*p*-toluenesulfonyloxyheptan-6-ol (1.42 g) in 10 ml diethyl ether was added dropwise over 20 min to the stirred suspension. The mixture was stirred for 2 hr, followed by refluxing for 3 hr. After cooling to 0°C, 2 N aq. NaOH (2.4 ml) was added, and the mixture was stirred for 2 hr. After removal of the aqueous layer and drying of the organic layer with anhyd. $MgSO_4$, the solvent was removed *in vacuo*. Chromatography on silica gel (32–63 μm, 60 Å, ethyl acetate/hexane 1:4) yielded 789 mg (90%) (2*RS*,5*S*)-5-ethyl-6-methylheptan-2-ol **5** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.83/0.84 (2 d, 6 H, J=6.6 Hz, CH(CH_3)$_2$), 0.86 (*t*, 3 H, J=7.4 Hz, CH_2CH_3), 0.98–1.04 (m, 1 H, CH_2CHCH_2), 1.13–1.22/1.30–1.38 (2 m, 2 H, CH(OH)CH_2CH_2), 1.19 (d, 3 H, J=6.1 Hz, CH_3CHOH), 1.23–1.32 (m, 2 H, CH_2CH_3), 1.38–1.44 (m, 2 H, CH_2CHOH), 1.65–1.73 (m, 1 H, $CH(CH_3)_2$), 3.76 (m, 1 H, CHOH). ^{13}C NMR (101 MHz, $CDCl_3$): δ 12.19 (q, C9), 19.31/19.35/19.49/19.51 (2q, C7 and C10), 23.04 (t, C8), 23.60/23.66 (q, C1), 26.11/26.13 (t, C4), 29.09 (d, C6), 37.64 (t, C3), 45.69 (d, C5), 68.82/68.88 (d, C2). MS (70 eV), *m*/*z* (relative abundance): 39 (18), 40 (4), 41 (64), 42 (15), 43 $\left(88, CH_3CHCH_3^+\right)$, 44 (10), 45 (99, CH_3CHOH^+), 53 (6), 55$\left(100, C_4H_7^+\right)$, 56 (16), 57 (17), 58 (4), 59 (7), 67 (3), 69 (37), 70 (18), 71 (9), 81 (4), 83 (9), 84 (12), 85 (6), 97 (26, M^+-iPr, -H_2O), 98 (11), 115 (3), 123 (1), 125 (2, M^+-H_2O, -Me), 140 (2, M^+-H_2O), 141 (2).

(S)-3-Ethyl-4-methylpentan-1-ol, (S)-1 Powdered 4-Å molecular sieve (1.28 g) was added to a solution of (2*RS*,5*S*)-5-ethyl-6-methylheptan-2-ol **5** (638 mg, 4.03 mmol) in 30 ml dry CH_2Cl_2 under nitrogen. The vigorously stirred suspension was cooled to −10°C, and pyridinium dichromate (3.2 g, 8.5 mmol) was added in small portions. Stirring then was continued for 24 hr at room temperature. After addition of 30 ml diethyl ether, the mixture

was filtered through a plug of silica gel. After washing the filter cake with several portions of diethyl ether, drying of the combined ethereal solution with anhyd. $MgSO_4$ and removal of the solvent *in vacuo*, 611 mg of crude (*S*)-5-ethyl-6-methylheptan-2-one were obtained as a colorless oil. The mass spectrum of the compound was in accord with that previously reported (Renold et al. 1974), and the compound was used without further purification.

Aqueous NaOH (20 ml) was added to a stirred solution of 375 mg of crude (*S*)-5-ethyl-6-methylheptan-2-one in 15 ml methanol, followed by 20 ml of 30% aqueous hydrogen peroxide in four portions over 5 d while stirring in the dark. The mixture then was heated to 50°C and small portions of an aqueous solution of sodium sulfite were added until the remaining peroxide was destroyed (no darkening of an acidic solution of potassium iodide). After cooling, the reaction mixture was saturated with sodium chloride and extracted five times with 25 ml diethyl ether. The combined extracts were washed twice with brine, dried over anhyd. $MgSO_4$, and concentrated *in vacuo*. Silica gel chromatography, eluting with ethyl acetate/hexane 1:4 yielded 54.6 mg (0.42 mmol, 17%) of (*S*)-3-ethyl-4-methylpentan-1-ol, (*S*)-**1**, of 98% ee, as a colorless oil. Spectroscopic data matched those of the racemic material and those reported in the literature (Enders and Rendenbach 1986).

(R)-4-(1-Methylethyl)tetrahydropyran-2-one (**7**) (*R*)-(+)-dihydrolimonene **3** (see above) was converted to methyl (*R*)-3-(1-methylethyl)-6-oxoheptanoate **6** as described by Kitahara and Mori (1984). A mixture of methyl (*R*)-3-(1-methylethyl)-6-oxoheptanoate **6** (1.24 g, 6.19 mmol), 60 ml methanol, 24 ml 10% aq. NaOH, and 56 ml of 30% aq. H_2O_2 was stirred for 3 d in the dark. Work-up and purification as described for the synthesis of (*S*)-**1** from (*S*)-5-ethyl-6-methylheptan-2-one (see above) yielded 475 mg (3.34 mmol, 54%) of (*R*)-4-(1-methylethyl) tetrahydropyran-2-one **7**. Analytical data matched literature values (Theisen and Heathcock 1993).

Methyl (S)-5-bromo-3-(1-methylethyl)pentanoate (**8**) A solution of of (*R*)-4-(1-methylethyl)tetrahydropyran-2-one **7** (451 mg, 3.17 mmol) in 35 ml methanol was cooled to −30°C, then a gentle stream of dry HBr gas was passed into the stirred solution. After termination of the reaction (approximately 1 hr), the mixture was added slowly to 50 ml of ice-cold brine. The mixture was extracted with 15 ml diethyl ether, and the layers were separated. The aqueous layer was extracted three more times with 30 ml diethyl ether. The combined organic extracts were washed twice with saturated aq. $NaHCO_3$ and then with 8 ml of brine. The solution was dried over anhyd. $MgSO_4$, and the solvent was removed *in vacuo*. Silica gel chromatography, eluting with ethyl acetate : hexane, 1:6, yielded 623 mg (2.63 mmol, 83%) of bromide **8** as a slightly yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87/0.90 (2 d, 6H, J=6.6 Hz, $CH(CH_3)_2$), 1.72–1.77 (m, 1 H, $CH(CH_3)_2$), 1.75–1.82/1.89–1.99 (2 m, 2H, $BrCH_2CH_2$), 1.89–1.99 (m, 1 H, CH_2CHCH_2), 2.18/2.34 (dd, J=15.4/7.4 Hz, dd, J= 15.4/5.9 Hz, 2H, CH_2COO), 3.45–3.67 (m, 2 H, CH_2Br), 3.68 (s, 3 H, $COOCH_3$). ^{13}C NMR (101 MHz, $CDCl_3$): δ 18.60/19.35 (q, C-7 and C-8), 29.94 (d, C-6), 31.77 (t, C-5), 34.81 (t, C-4), 35.47 (t, C-2), 39.93 (d, C-3), 51.77 (q, C-9), 173.89 (s, C-1). MS (70 eV), *m/z* 39 (31), 40 (6), 41 (78), 42 (15), 43 (80), 44 (3), 45 (6), 51 (4), 52 (2), 53 (21), 54 (11), 55 (99), 56 (7), 57 (8), 59 (50), 65 (2), 67 (9), 68 (2), 69 (29), 70 (3), 71 (13), 72 (3), 73 (11), 74 (100), 75 (8), 79 (4), 81 (17), 82 (10), 83 (57), 84 (4), 85 (2), 87 (42), 88 (4), 93 (2), 95 (3), 96 (2), 97 (30), 98 (2), 99 (4), 101 (11), 107 (4), 109 (7), 113 (4), 115 (4), 121 (2), 123 (2), 125 (27, 205-HBr), 126 (2), 129 (4), 135 (2, $C_4H_8Br^+$), 137 (1),141 (3), 143 (1), 157 (12, M^+-Br), 161 (2), 163 (16), 164 (2, M^+-88), 165 (14), 187 (1, 205-H_2O), 189 (1), 193 (27, M^+-iPr), 194 (3), 195 (26), 196 (3), 205 (7, $^{79}M^+$-OMe), 207 (7), 221 (0.6,$^{79}M^+$-Me), 223 (0.6), 236 (0.2, $^{79}M^+$), 237 (0.3, $^{79}M^+$+1), 238 (0.2), 239 (0.3).

(R)-3-Ethyl-4-methylpentan-1-ol, (R)-1 A solution of bromide **8** (103 mg, 0.44 mmol) in 15 ml dry diethyl ether was added dropwise to a stirred suspension of $LiAlH_4$ (35 mg, 0.91 mmol) in 15 ml dry diethyl ether. The mixture was stirred for 2 hr, then refluxed 3 hr. Work-up and purification as described for the syntheses of **5** (see above) yielded 53 mg (0.40 mmol, 92%) of (*R*)-**1**, 97% ee. Spectroscopic data matched those of (*S*)-**1**.

Field bioassays

Field trials were conducted in July of 2004 and 2005. Solutions of test compounds in hexane were loaded onto 11-mm gray rubber septum lures (West Pharmaceutical Services, Lionville, PA, USA). Septa treated only with hexane were used as controls. Lures were placed in the centers of Pherocon® AM sticky traps, and traps were placed on the ground. A replicate consisted of the test treatments plus a control, each on its own sticky trap. The placement of the traps within a replicate was randomized, with the traps spaced 3 m apart. Replicates were spaced ~50 m apart and at least 15 m from the nearest *P. breviceps* nest. Traps were set out at 1600 hour and retrieved 2 hr later. Trap count data were square-root transformed ($X+0.5$) and analyzed by analysis of variance (ANOVA), followed by Student–Newman–Keuls tests to determine differences between means ($\alpha=0.05$).

Results

The syntheses of (*S*)-(-)-3-ethyl-4-methylpentan-1-ol, (*S*)-**1** and its enantiomer started from (*R*)-(+)-limonene **2** (Fig. 1). Commercially available **2** was hydrogenated to (*R*)-(+)-dihydrolimonene **3** as described by White et al. (1981). This was then transformed to either (3*R*,6*RS*)-3-(1-methylethyl)heptan-1,6-diol **4** (Nicotra et al. 1986) or to methyl (*R*)-3-(1-methylethyl)-6-oxoheptanoate **6** (Kitahara and Mori 1984), the respective precursors for each of the two enantiomers.

For the synthesis of (*S*)-**1**, the primary hydroxyl group of diol **4** was selectively tosylated, followed by reduction of the tosylate to (2*RS,5*S)-5-ethyl-6-methylheptan-2-ol **5** with $LiAlH_4$ (analogous to Kishida 1960). Oxidation of **5** to (*S*)-5-ethyl-6-methylheptan-2-one with pyridinium dichromate followed by Baeyer–Villiger oxidation yielded the target compound, (*S*)-**1**, with 98% ee.

For the synthesis of (*R*)-1, ketoester **6** was transformed to (*R*)-4-(1-methylethyl) tetrahydropyran-2-one **7**. Treatment of **7** with dry HBr in methanol (Paquette et al. 1991) yielded methyl (*S*)-5-bromo-3-(1-methylethyl)pentanoate **8**, which was converted to (*R*)-**1** of 97% ee by reduction with $LiAlH_4$ (Jefford et al. 1972).

Analysis of the volatiles from squashed heads of virgin queens on a Cyclodex-B chiral stationary phase GC column showed that queens produced exclusively the (*R*)-enantiomer (Fig. 2). This result was confirmed in field bioassays. In the first field trial in 2004, using blends of methyl 6-methylsalicylate with racemic and enantiomerically enriched (*R*)- and (*S*)-3-ethyl-4-methylpentanol (89 and 86.4% ee, respectively), mixtures of the racemic or the (*R*)-alcohol plus the salicylate were highly attractive to male ants, whereas the (*S*)-enantiomer plus the salicylate blend was much less attractive (Fig. 3A). The weak attraction to the latter blend may have been caused by the small percentage of the contaminating (*R*)-enantiomer in the blend, so the bioassay was repeated in 2005 with newly synthesized compounds of much higher enantiomeric purity ((*R*), 97% ee, (*S*), 98% ee). With these almost pure enantiomers, the results were unequivocal: traps baited with blends of the

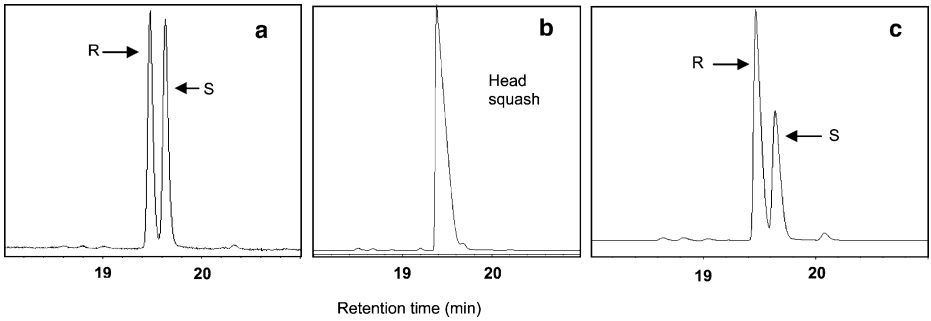


Fig. 2 Gas chromatograms on a Cyclodex B column of: (a) synthetic racemic 3-ethyl-4-methylpentanol; (b) SPME-collected volatiles from a *P. breviceps* queen head squash; c) volatiles from a queen head squash spiked with racemic 3-ethyl-4-methylpentanol, showing the enlargement of the first (3*R*) peak. Chromatography conditions are described in "Methods and Materials"

racemic or (*R*)-alcohol were highly attractive to male ants, whereas the corresponding blend prepared with the (*S*)-enantiomer attracted no male ants at all (Fig. 3B).

Discussion

There are about 190,000 described species within the order Hymenoptera, which includes the ants, bees, wasps, and sawflies (Goulet and Huber 1993). Within this group, numerous researchers have described anecdotal evidence for the use of sex pheromones and attractants, but sex pheromones have been conclusively demonstrated with bioassays in few cases (reviewed in Ayasse et al. 2001). The dearth of data on mating behavior in this group to some extent reflects the difficulty in observing and studying insects that often mate in flight or under circumstances that render detailed observation and manipulation difficult. Among the ants (family Formicidae), which consist of over 10,000 described species in 16 extant subfamilies (Bolton 1995), evidence of sex pheromones or attractants secreted by males or females has been described in three subfamilies: the Ponerinae, Myrmicinae, and Formicinae. For example, *Rhytidoponera* females (Ponerinae) release a sex attractant from their abdominal pygidial glands (Meinwald et al. 1983), whereas *Pogonomyrmex* males (Myrmicinae) release 4-methyl-3-heptanone from their mandibular glands, which attracts both sexes (McGurk et al. 1966). In the subfamily Formicinae, methyl 6-methylsalicylate, the most abundant component we identified in extracts of heads of *P. breviceps* queens, is also found in mandibular glands of males of several *Camponotus* species (Hölldobler and Maschwitz 1965; Torres et al. 2001), and its release has been shown to trigger flight of sexuals from the nest (Hölldobler and Maschwitz 1965). This same compound has also been identified from a ponerine ant, *Gnamptogenys pleurodon* (Duffield and Blum 1975) and as a trail pheromone in the myrmicines *Tetramorium impurum* (Morgan and Ollett 1987) and *Mayriella overbecki* (Kohl et al. 2000). The second component of the sex pheromone of *P. breviceps*, 3-ethyl-4-methylpentanol, has been identified previously from mandibular glands of workers of *Harpegnathos saltator* (do Nascimento et al. 1993) and in heads of *Formica rufa* workers and *Formica polyctena* queens (Francke et al. 1985). However, the biological relevance of this compound for the latter three species has not been reported, nor has the absolute configuration of the compound from these various species been determined.

In *Formica lugubris*, the only other ant for which a sex pheromone has been identified (Walter et al. 1993), males and females fly from their nests to open places in the forest

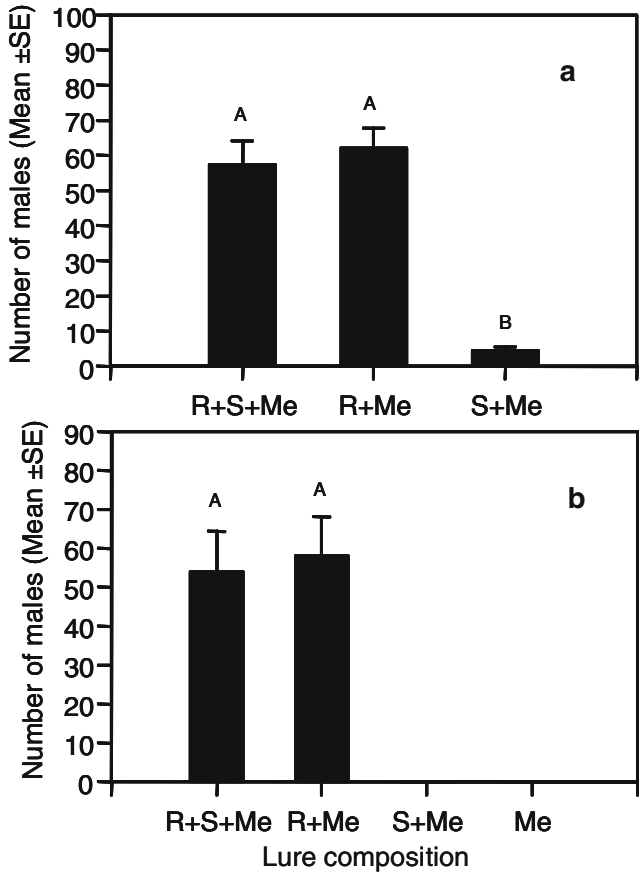


Fig. 3 Mean numbers (± SE) of *P. breviceps* males captured in traps baited with mixtures of racemic or enantiomerically enriched (*R*)- and (*S*)-3-ethyl-4-methylpentanol with 6-methylsalicylate. "R"=(*R*)-enantiomer, "S"=(*S*)-enantiomer, and "Me"=methyl 6-methylsalicylate. Trial 1 with less pure enantiomers (a): blend ratios were racemic (R+S)/Me (25:75), R/Me (15:75), and S/Me (15:75). Trial 2 with high enantiomeric purity materials (b): blend ratios were racemic (R+S)/Me (25:75), R/Me (12.5:75), and S/Me (12.5:75). Means with the same letter are not significantly different (Newman–Keuls test on square root transformed count data)

where they mate on the ground. Extracts of the Dufour's gland were found to contain three volatile compounds (undecane, tridecane, and (*Z*)-4-tridecene, mean ratio 100:5:4), but undecane as a single component was as attractive as the three compounds together. Thus, even within the same ant subfamily, the sex pheromone system in this species bears no relationship to that of *P. breviceps*, with the pheromone being produced from an entirely different gland, and the chemistry of the pheromone components also being entirely different.

The sex pheromone of *P. rufescens*, a European congener of *P. breviceps*, was reported to consist of methyl 6-methylsalicylate as a single component (Castracani et al. 2005). Although it is difficult to directly compare their results with ours because of differences in bioassay designs (e.g., Castracani et al. 2005 placed traps only 2 m from nests, whereas ours were at least 15 m away), the relatively low trap catches they reported compared with ours suggest that the reported pheromone of *P. rufescens* may be incomplete.

Previous reports had suggested that the *P. breviceps* pheromone was produced in the mandibular glands (Topoff and Greenberg 1988; Greenberg et al. 2004). However, the squashes used in those studies did not distinguish between mandibular glands located in the head behind the mandibles and the newly described intramandibular glands (Grasso et al. 2004). Therefore, it is possible that one or both of the two pheromonal components come from the intramandibular glands.

Polyergus breviceps and *P. rufescens* both illustrate the "female calling syndrome" (Hölldobler and Bartz 1985; Ayasse et al. 2001), with queens remaining close to their nest of origin where they emit sex attractant pheromones. This behavior may be adaptive for parasitic ants that are uncommon or that live in patchy environments. This mating strategy assures that queens mate in the habitat that contains the slave species that are essential for starting new colonies. Other ant species such as harvester ants in the genus *Pogonomyrmex* (Hölldobler and Bartz 1985; Ayasse et al. 2001) use a different mating strategy called the "male-aggregation syndrome," whereby unmated female reproductives fly upwind to aggregations of pheromone-producing males.

Pheromones consisting of blends can increase the message specificity created from subsets of a relatively small number of compounds. The examples mentioned above suggest that volatile components used for communication by ants are quite highly conserved, with the same compounds being found repeatedly in even quite distantly related species. Reconstruction of the sex pheromones of other ant species may be facilitated by behavioral bioassays conducted in tandem with gas chromatography-electroantennogram detection (GC-EAD) analysis, as proved to be crucial in the location and identification of the minor component of the *P. breviceps* pheromone (Greenberg et al. 2004). Furthermore, the specificity of a sex pheromone blend is likely to be important in reproductive isolation of closely related sympatric species (Blum 1981). We plan to test this prediction in ongoing research comparing the sex pheromone blend of *P. breviceps* with that of a second slave-making ant, *P. lucidus*, in their areas of sympatry in the United States.

Acknowledgments This work was supported in part by Hatch Act funding to J.G.M., #CA-R*ENT-5181-H.

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J Chem Ecol (2007) 33:947–961
DOI 10.1007/s10886-007-9277-2

Molecular Characterization of Two Pheromone Binding Proteins and Quantitative Analysis of their Expression in the Beet Armyworm, *Spodoptera exigua* Hübner

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Received: 28 September 2006 / Revised: 23 January 2007 / Accepted: 27 February 2007 /
Published online: 29 March 2007
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Abstract Pheromone binding proteins (PBP) play an important role in insect pheromone communication. However, the PBP for the beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), an important agricultural pest worldwide, remains unaddressed. We report the cloning of two PBP genes, *SexigPBP1* and *SexigPBP2*, from the antennal cDNA of *S. exigua* by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends-PCR (RACE-PCR). The deduced PBP amino acid sequences are characteristic of the odorant binding protein (OBP) family, although the two PBPs are only 44% identical. From an analysis of the genomic DNA, two introns and a similar intron/extron structural pattern were identified in each of the two PBP genes. RT-PCR analysis revealed that the two PBP genes are only expressed in antennae. Real-time PCR further indicated that the expression of *SexigPBP1* is much higher than that of *SexigPBP2*, regardless of sex. However, the female expression levels for *SexigPBP1* and *SexigPBP2* are about 39% and 73%, respectively, relative to male levels. Finally, phylogenetic analysis suggested that PBPs from the Noctuidae are divided into three distinct groups based on the primary sequences.

Keywords Anatomical expression · Cloning · Expression level · Pheromone binding protein · Rapid amplification of cDNA ends (RACE) PCR · Real-time PCR · Reverse transcription (RT) PCR · *Spodoptera exigua*

Introduction

Interactions of insects with their surroundings are based primarily on chemical signals. Odor reception in insects involves small (15–17 kDa) soluble proteins, called odorant

Electronic supplementary material The online version of this article (doi:10.1007/s10886-007-9277-2) contains supplementary material, which is available to authorized users.

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binding proteins (OBPs) (Vogt and Riddiford 1981). OBPs are synthesized by accessory cells and accumulated to high concentrations (10 mM) in the sensillar lymph (Steinbrecht et al. 1992). They are postulated to solubilize semiochemicals, to protect the ligands from degradation, and to participate in fast inactivation of chemical signals (Kaissling 1986, 1996; Du and Prestwich 1995; Prestwich et al. 1995; Klusák et al. 2003). Insect OBPs are all α-helical proteins (Sandler et al. 2000), thus, completely different from mammalian OPBs, which belong to the lipocalin superfamily and have a β-barrel fold (Spinelli et al. 1998; Tegoni et al. 2000). OBPs can be further divided into two classes: highly specific and sensitive pheromone binding proteins (PBPs) and less specific general odorant binding proteins GOBP1 and GOBP2 (Vogt and Riddiford 1981; Robertson et al. 1999; Vogt et al. 1999).

In moths, the pheromone communication system is extremely sensitive, enabling the detection of a few molecules, and selective in the discrimination between closely similar compounds or isomers (Hansson 1995). PBPs have been identified from various moth species (Vogt and Riddiford 1981; Maïbèche-Coisné et al. 1998; Robertson et al. 1999; Picimbon and Gadenne 2002), and the proteins display considerable diversity, sharing 32–92% amino acid identity (Abraham et al. 2005). Moreover, there is evidence for multiple PBPs in a single moth species, each encoded by a distinct locus (Bette et al. 2002; Picimbon and Gadenne 2002). Ligand-binding experiments in some moths, especially in the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), have shown that the various PBPs show specificity in binding their respective pheromones (Du and Prestwich 1995; Plettner et al. 2000; Bette et al. 2002; Maida et al. 2003). Therefore, it has been suggested that PBPs in some species may be involved in discriminating between pheromone components.

The beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae), is a severe pest of various agricultural crops in Asia and North America. The sex pheromone of female *S. exigua* has been identified as a complex blend, and a lure with two components (*Z*9,*E*12–14:Ac and *Z*9–14:OH) has been successfully used in pest population monitoring and management (Wakamura et al. 1989; Mitchell and Tumlinson 1994; Yoshiyasu et al. 1995; Mitchell et al. 1997; Dong and Du 2002). However, the molecular mechanism of the perception of female sex pheromone by the male in *S. exigua* remains unknown. Here, we report the molecular isolation and identification of two genes from *S. exigua* that encode members of the PBP family. In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to investigate the distribution of PBPs in different tissues, and the mRNA expression levels of both PBPs in antennae were detected in male and female moths by real-time PCR.

Methods and Materials

Collection of Tissues

Spodoptera exigua were reared at 27°C (14:10 L:D) in the laboratory on an artificial diet (Huang et al. 2002) and sexed as pupae. Antennae were collected by excising the tissue at the base from 3-d-old male and female moths and immediately transferring it to Eppendorf tubes immersed in liquid nitrogen. The same procedure was used for collecting the head (without antennae), thorax, abdomen, and legs. All tissues were stored at −75°C until used experimentally.

Nucleic Acid Manipulation

Total RNA was extracted by homogenizing antennae or other tissues of male and female moths in Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's

instructions. Total RNA was transcribed into single strand cDNA that served as templates for PCR amplification by extension of an oligo(dT)$_{18}$ primer using MMLV reverse transcriptase (Promega, Madison, WI, USA) at 42°C for 60 min. The reaction was stopped by heating at 95°C for 5 min.

Genomic DNA was prepared from one-fourth instar. Animal tissues were disrupted by grinding in liquid nitrogen and homogenizing with 300 μl DNA extraction buffer (100 mM Tris–HCl, pH 8.0; 50 mM ethylenediaminetetraacetic acid (EDTA); 200 mM NaCl; 1% SDS). The homogenate was transferred to an Eppendorf tube, gently mixed with 20 μl Proteinase K (20 mg/ml), and incubated at 56°C for 3 hr. The tube was centrifuged for 10 min at 12,000×*g*. The supernatant was transferred to a new tube. DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by an equal volume of chloroform:isoamyl alcohol (24:1), each time centrifuging for 5 min at 12,000×*g* to collect the aqueous phases. Two-and-one-half volumes of chilled ethanol were added to the aqueous supernatant for DNA precipitation. The pellets were washed in 70% ethanol, briefly vacuum-dried, and the DNA was redissolved in 50 μl Tris–EDTA (TE) buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0; 100 μg/ml RNase A).

PCR and Sequencing

The cDNAs encoding pheromone binding proteins of *S. exigua* were amplified from antennal cDNA of males. Comparing PBP sequences from *Heliothis virescens* (Krieger et al. 1993, GenBank X96861), *Helicoverpa zea* (Callahan et al. 2000, GenBank AF090191), *Agrotis ipsilon* (Picimbon and Gadenne 2002, GenBank AY301986), *Agrotis segetum* (LaForest et al. 1999, GenBank AF134253), and *Mamestra brassicae* (Maïbèche-Coisné et al. 1998, GenBank AF051142 and AF051143), a pair of degenerate primers (Table 1) was designed to match amino acid regions of FWK(R)EG(E)Y and HE(D)LN(K)WA. Amplification was carried out in a PTC-200 (MJ Research, Waltham, MA, USA) and included negative controls under the following conditions: 94°C for 3 min; 9 cycles at 94°C for 50 sec, 65°C for 1 min, and 72°C for 30 sec, with a decrease of the annealing temperature by 1°C per cycle. This was followed by 25 cycles at 94°C for 50 sec, 55°C for 1 min, and 72°C for 30 sec, and final incubation for 10 min at 72°C. The reaction was performed in 50 μl with 3 μl single-stranded cDNA, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 1.5 μM for each primer and 2.5 U Taq polymerase (Promega).

A RACE procedure was employed to amplify the 5′ and 3′ end of the coding region using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) following the kit instructions. Gene-specific primers (GSP) (Table 1) for 5′-RACE and 3′-RACE were derived from the sequence of PCR products. Amplification conditions were 94°C for 2 min; 28 cycles at 94°C for 30 sec, 68°C for 30 sec, and 72°C for 3 min.

Regions of the PBP gene were amplified from genomic DNA by PCR to ascertain the intron/exon structure of the genes. Specific primer pairs (Table 1) were chosen for the amplification. Primer sequences were designed to include a *Bam*HI restriction site in the sense primers and an *Xho*I restriction site in the antisense primers (underlined). Touchdown PCR reactions were performed as follows: 94°C for 3 min, 5 cycles at 94°C for 50 sec, 61°C for 50 sec, and 72°C for 2 min, with a decrease of the annealing temperature by 1°C per cycle. Subsequently, 35 cycles of 94°C for 50 sec, 55°C for 50 sec, and 72°C for 2 min was followed by incubation for 10 min at 72°C. LA Taq polymerase (LA Taq Polymerase is a thermostable polymerase that possesses a 3′ to 5′ exonuclease activity, allowing amplification of long targets, TaKaRa, Otsu, Shiga, Japan) was used following manufacturer's instructions.

Table 1 Oligonucleotide primers used for isolation and expression analysis of *Spodoptera exigua* pheromone binding proteins (PBP)

Purpose/Primer Name	Sequence (5′–3′)
cDNA Isolation (RT-PCR)	
Sense	TTYTGGMRNGARGRNTA
Antisense	GCCCANTTNARNTCRTG
5′ and 3′ cDNA End Isolation (RACE)	
SexigPBP1	
5′GSP[a]	TCTTGGTGCGGAAGCATTTGGATA
3′GSP	CTGATGACGAGGTTGCCAAGAAGC
SexigPBP2	
5′GSP	GCACTTGGCTACATTCAGGGTCTTCAT
3′GSP	CGGCTGTGCTATCCTGTGTCTCTCTTC
Genomic DNA Isolation	
SexigPBP1	
Sense	A*GGATCC*ATGGCGGGCGCAAAAT
Antisense	A*CTCGAG*CTACACGGCGGTCATCACC
SexigPBP2	
Sense	A*GGATCC*ATGGCGTTCTGTCGATCAGCG
Antisense	A*CTCGAG*TTACACTTCAGCCAGCACTTCTCCC
Tissue Localization (RT-PCR)	
SexigPBP1	
Sense	CGGTTTGTCTGTGTTGTGTTCG
Antisense	TTTCCTGGTTCCCTCGTGTCTA
SexigPBP2	
Sense	GCGAAACCTTTGGATGACT
Antisense	AGCCTACTACTACCCACACCC
ACTB	
Sense	ATCTGGCACACCTTCTACAACGA
Antisense	TCACGCACGATTTCCCTCTCA
Expression analysis (real-time PCR)	
SexigPBP1	
Sense	TGGCAAAGAAGTTGGACC
Antisense	TGCTCGCATTCGTGGAT
SexigPBP2	
Sense	CAGTATGCTGGTAGTGGTA
Antisense	GAAGTTGTAGAAGTCGGTC
ACTB	
Sense	ATCCTCCGTCTGGACTTGG
Antisense	CGCACGATTTCCCTCTCA

[a] Gene-specific primer

PCR products were analyzed by 1.5% agarose-gel electrophoresis, and DNA purification was performed using Wizard PCR Preps DNA purification kit (Promega). Purified products were subcloned in a T/A cloning plasmid using the pGEM-T easy vector system (Promega) following manufacturer's instructions. The plasmid DNA was transformed into DH5α competent cells. Positive clones on the basis of restriction enzyme cleavage sites and PCR amplification were sequenced using dye-terminator chemistry and reactions on an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence Analysis

Sequences were identified with the NCBI-BLAST network server (Altschul et al. 1990). The putative signal peptides and their cleavage sites were predicted by using the SignalP V 3.0 program (Bendtsen et al. 2004; http://www.cbs.dtu.dk/services/SignalP/). The hydrophobicity profile was determined by the method of Kyte and Doolittle (1982). The sequence data were aligned and compared by using Clustal X (1.81) (Thompson et al. 1997). The phylogenetic tree was constructed with Neighbor Joining (NJ) by the MEGA2.1 program (Kumar et al. 2001). Bootstrap analysis used 1,000 replications.

Tissue Specificity of SexigPBP

Tissue specificity was assessed by reverse transcription (RT)-PCR. RT-PCR templates were the cDNA from antennae and other tissues of male and female moths, and genomic DNA. Specific primer pairs (Table 1) were derived from the cDNA sequences, spanning at least one predicted intron region, thus, allowing us to distinguish amplification products resulting from cDNAs templates from those amplified from possible genomic contaminants in the cDNA preparation. For testing the integrity of the cDNA templates, a control primer pair (Table 1) from the coding region of the *S. exigua* β-actin gene (*ACTB*) (GenBank AY507963) was used. PCR began at 94°C for 3 min, then 5 cycles at 95°C for 50 sec, 54°C for 50 sec, and 72°C for 2 min (with a decrease of the annealing temperature by 1°C per cycle); followed by 35 cycles at 95°C for 50 sec, 49°C for 50 sec, and 72°C for 2 min; with a final 10-min incubation at 72°C.

Real-time PCR Analysis of SexigPBP Expression

Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems,) by using SYBR green dye bound to double strand DNA at the end of each elongation cycle (SYBR Green Realtime PCR Master Mix; TOYOBO, Osaka, Japan). PCR primers were designed to yield products of 75 to 150 bp; each pair was designed to span a cDNA exon–exon border to avoid amplification of potential traces of genomic DNA (Bohbot and Vogt 2005). SexigPBP1 primers (Table 1) amplified a 130-bp product from cDNA. SexigPBP2 primers (Table 1) amplified a 133-bp fragment from cDNA. As an endogenous control to normalize the results of a variable target gene and to correct for sample-to-sample variation, the amplification size of *ACTB* primers (Table 1) was 107 bp.

Real-time PCR was conducted in 25 μl reactions containing 12.5 μl of 2× SYBR Green PCR Master Mix, 0.5 μl of each primer (10 μM), 2.5 μl of sample cDNA, and 9 μl sterilized ultrapure H_2O (Millipore). To check reproducibility, test samples, endogenous control, and "no sample control" were done in triplicate. Cycling parameters included 94°C for 3 min, 40 cycles at 94°C for 30 sec, and 62°C for 45 sec. The products were analyzed by sequencing, agarose gel electrophoresis, and melting curve, which indicated that each reaction did not produce nonspecific amplification.

Relative quantification was performed by using the comparative $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) to identify the amount of PBP mRNA in female antennal tissue relative to the amount in male tissue. All data were normalized to endogenous *ACTB* levels from the same individual sample. In the analysis of the relative fold change, the male sample, which was one of the experimental samples, was taken as the calibrator (Mittapalli et al. 2006). Thus, the relative fold change was assessed by comparing the expression level

of *SexigPBP* in female moths to that in males. Calculations for relative quantification were performed according to the instructions of the User Bulletin #2 from Applied Biosystems (Relative Quantification of Gene Expression; ABI Prism 7700 Sequence Detection System).

An assumption in the $\Delta\Delta C_T$ calculation for the comparative $2^{-\Delta\Delta C_T}$ method for relative quantification is that the amplification efficiencies of the target and reference are approximately equal. To confirm this, a pilot experiment was conducted to examine the variation of ΔC_T ($C_{T,\text{Target}} - C_{T,ACTB}$) with template dilution. Briefly, four serial tenfold dilutions of cDNA from each sample were amplified. For each dilution, amplifications were performed in triplicate by using primers for *PBP* and *ACTB*. Mean C_T was calculated for both *PBP* and *ACTB*, ΔC_T was calculated, and log cDNA dilution vs. ΔC_T was plotted.

```
   1 ACGCGGGGGCAGATAACAAGATGGCGGGCGCAAAATGGCGGTTTGTCTGTGTTGTGTTCGCGCTGTACCTGACCAGCGCC
                     M  A  G  A  K  W  R  F  V  C  V  V  F  A  L  Y  L  T  S  A
                                                                          ><1
  81 GCGCTGGGCTCGCAGGAGCTCATGATGAAGATGACTAAGGGATTCACGAAAGTCGTCGATGAGTGCAAAGCTGAGCTTAA
     A  L  G  S  Q  E  L  M  M  K  M  T  K  G  F  T  K  V  V  D  E  C  K  A  E  L  N
 161 CGCGGGGGAGCACATCATGCAGGACATGTACAACTACTGGCGCGAAGACTACCAGCTCATTAACCGGGACTTGGGCTGCA
      A  G  E  H  I  M  Q  D  M  Y  N  Y  W  R  E  D  Y  Q  L  I  N  R  D  L  G  C  M
 241 TGATCCTGTGCATGGCAAAGAAGTTGGACCTCATCGAAGACCAGAAGATGCCACCACGCGAAGACAGAAGAATTCGCCAAG
       I  L  C  M  A  K  K  L  D  L  M  E  D  Q  K  M  H  H  G  K  T  E  E  F  A  K
                  ><2
 321 AGTCATGGCGCTGATGACGAGGTTGCCAAGAAGCTGGTGAGCATAATCCACGAATGCGAGCAGCAGCACGCCGGCATAGC
      S  H  G  A  D  D  E  V  A  K  K  L  V  S  I  I  H  E  C  E  Q  Q  H  A  G  I  A
 401 GGACGATTGCATGAGGGTGTTGGAGATATCCAAATGCTTCCGCACCAAGATTCACGAGCTCAAATGGGCACCCAACATGG
      D  D  C  M  R  V  L  E  I  S  K  C  F  R  T  K  I  H  E  L  K  W  A  P  N  M  E
 481 AGGTCATTATGGAAGACGGTGATGACCGCCGTGTAGACACGAGGGAACCAGGAAACAATGTCATTTTAGGGAAAACTGCTG
       V  I  M  E  E  V  M  T  A  V  *
 561 CAGTTGTTGGAGTGTCACGCGGGATAATGATCTGCAGCGTTAGCAAAACTGATGTACATACTTGTAATCGAGAATGCTAT
 641 GGCAAACGAAACAAATGTATTTGGAGATTTATCAGTTTGAATACGTTGTGTGGCGCGTGGGCAATAGTGAATCTATACGT
 721 ATGAACAACATTTGTTTCCTTTTATTTAGCGTTAACGATCACAAGTTGTACTGAACGATAACTAAAGCTCATAATGGTTC
 801 TAAGATTATCTCTAGATTGCAGGGCTATAACTGGAAAGGGTTTCGTGTCATTTCGTTTCATGTCGCTGATTGATCAACAC
 881 TTTCTAACACCTTTACACAATTCTCTTCAATCGTCGGAGTTATTCTACTTCACCCAGAAGTGAAATTGTTGTATCATTAT
 961 CCTGGCTCTTTATTCAGTGAAACTATGTAGCTGTATAAGTATTATTTATTTCCTCTTTAGGTTCTTGGTTAATTAAAGTG
1041 TTTCAATTCATGAAAAAAAAAAAAAAAAAAAAAAAAA
```

Intron1:

GTAAGATTATTTATAAAATCATTCCTTTTATTTGATAAAGAAGGTTTCTTTACATTGATGTTTATAAATACTTACTTTCTCTCGCAG

Intron2:

GTAACTTATCCTTGATATACTTTTCATCGCTTAGTACAAACGATGGTTTTCTCATCAAACATTTGATAGAAATAGAAGAAAATTGTTTACGT

CGAAAATTGAAAACTATCAAATACTGTGAAAATTAATCTTTTCTCATCAG

Fig. 1 cDNA sequence and predicted amino acid sequence of SexigPBP1. The stop codon is indicated with an *asterisk*. The signal peptide is *underlined*. Sites of intron one and two are marked with >< above the sequence, and the intron sequences are given at the *bottom* of the figure

```
   1  GGCCACAGCTTCTAATTAAGACGGCGATTAGCCGCGGATATAAGCGAGTCCTGTCGATGTTGCTGCCACAATGGCGTTCT
                                                                      M  A  F  C
  81  GTCGATCAGCGACCATGTCGGTGAGGGTGGCGCTGGTGGTGGCCGCCAGTATGCTGGTAGTGGTGCAGGCGTCGCAAGAT
       R  S  A  T  M  S  V  R  V  A  L  V  V  A  A  S  M  L  V  V  V  Q  A  S  Q  D
                                                          ><1
 161  GTCATGAAGAACTTGGCCATCAATTTCGCGAAACCTTTGGATGACTGTAAAAAGGAGATGGACCTGCCGGACTCTGTGAC
       V  M  K  N  L  A  I  N  F  A  K  P  L  D  D  C  K  K  E  M  D  L  P  D  S  V  T
 241  GACAGACTTCTACAACTTCTGGAAGGAAGGCTACGAGCTGACGAACAGACAGACCGGCTGTGCTATCCTGTGTCTCTCTT
       T  D  F  Y  N  F  W  K  E  G  Y  E  L  T  N  R  Q  T  G  C  A  I  L  C  L  S  S
                                                                                  ><2
 321  CGAAGTTGGAGATTCTTGACCAAGAACTGAACCTGCATCATGGCAGGGCGCAAGAGTTTGCTATGAAACACGGCGCTGAC
       K  L  E  I  L  D  Q  E  L  N  L  H  H  G  R  A  Q  E  F  A  M  K  H  G  A  D
 401  GAGACCATGGCGAAGCAGATAGTGGACATGATCCACACGTGTGCGCAGTCCACTCCCGACGTGGCGGCGGACCCTTGCAT
       E  T  M  A  K  Q  I  V  D  M  I  H  T  C  A  Q  S  T  P  D  V  A  A  D  P  C  M
 481  GAAGACCCTGAATGTAGCCAAGTGCTTCAAGTTGAAGATACACGAGCTCAACTGGGCGCCCAGCATGGAGCTCATCGTGG
       K  T  L  N  V  A  K  C  F  K  L  K  I  H  E  L  N  W  A  P  S  M  E  L  I  V  G
 561  GAGAAGTGCTGGCTGAAGTGTAACTTGAATCACTCAAGACCTTTAAGCTGGCCTTCATTATGTGAGGTCTTCATAAACAT
       E  V  L  A  E  V  *
 641  ATCTTTGACGTCTCGGCTCGTTGAACGGACCCCAGATTAGGTTAGGTTGGTCGTGAAGCATCTCCTGGCTCGCCAGTTCG
 721  CCTCAGAGGGTGTGGGTAGTAGTAGGCTGCAGCAAGATGTCAAATATTGTTCAATATACTGTACATCATTAAAAAAAAAA
 801  AAAAAAAAAAAAAAAAAAAA
```

Intron1:

```
GTAAGTTTGGAAAGTGGATTTTACTACAAATATTTAAATGATGTATAATCTCGAAAGTTTTCCCGTAAACCGTTACCGTTTATGCTATGCGT
GTTTGCTGTTACCATTCTTAGGCACCTATTCAGTTGCCAGCAATATATGCGAATAGTTTCATTCTGCTATATTCCATCCATTATCTCTGAGT
TTTGTGACACCTACATAGTTGAATAATATTATATTATTCGGCGTTCGTTGATAGCTAGCTATCTGTAACACACACATAATAATTAAATATCG
GCTCGGCTCGTTATTGTAG
```

Intron2:

```
GTTAGAACTTCTTTCATTGCAGTAACTTAGTTTTTATGAATGCAGTTTTCCAATATCGGGTTCCGTATAAACATCTCATAAAGTACCATGTG
ATGGCTACAACACGTACCTCTCAGATTAGACTTGGCACCAAAGTCAAATGTACCTATTGCTTGTCAGCTAGTTTTTTTTTTTGAGAAGGTAG
ATATAATTGACTTCTATATCCCTAGAACCAGTGTATATATGTGTCATATTTTTTTTCTTTTAATGTATTTCATGTTACAAGATAATGATGCA
CTAACAAAGCAAACAACAACAAAGTTAACCTCAACTTAGAAGGGGAGAGTGATATAAAAATTAACATGAATCAGCGAAATTTCCGATCTGTT
GAAGAGGAAAATTGTAAAGAAAGTGTAATTTCACGGAGATCTTATTGGAAAATATCTCGTGGTAATGTCGTTCTAGGTCGATGCTTTAAAAC
AAATAGCTATTTAACTAGCACGATAGCTTGGATTTACTTTATTTTATTGTTGGTACTTAATAGGAGACCATTTCAACCATACACACTGCGCG
ACTATATATTGTTCCCAGTATATCTTCTAGCATGCATTACAGAATCCTTCTTTCGTAGTATTCAGTAAAGCATTTGCTCCCTGATCTTCCAG
```

Fig. 2 cDNA sequence and predicted amino acid sequence of SexigPBP2. The stop codon is indicated with an *asterisk*. The signal peptide is *underlined*. Sites of intron one and two are marked with >< above the sequence, and the intron sequences are given at the *bottom* of the figure

Results

Identification and Characterization of SexigPBPs

PCR performed with a pair of degenerate primers designed on the conserved amino acid regions of FWK(*R*)EG(*E*)Y and HE(D)LN(K)WA in PBPs of other insects, afforded two

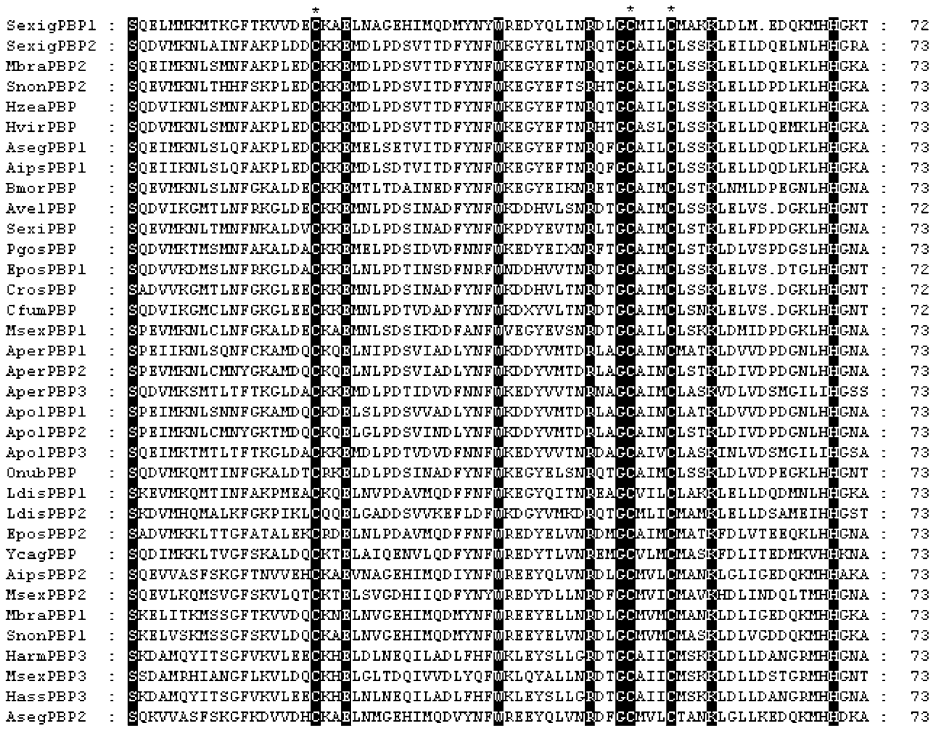


Fig. 3 Alignment of the mature SexigPBP1 and SexigPBP2 from *Spodoptera exigua* with their orthologs from other species of moths: SexigPBP1 and SexigPBP2 are at the top, AipsPBP1 (*Agrotis ipsilon*, AY301985), AipsPBP2 (*A. ipsilon*, AY301986), AperPBP1 (*Antheraea pernyi*, X96773), AperPBP2 (*A. pernyi*, X96860), AperPBP3 (*A. pernyi*, AJ277265), ApolPBP1 (*Antheraea polyphemus*, X17559), ApolPBP2 (*A. polyphemus*, AJ277266), ApolPBP3 (*Antheraea polyphemus*, AJ277267), AsegPBP1 (*Agrotis segetum*, AF134253–AF134294), AsegPBP2 (*A. segetum*, AY301987), AvelPBP (*Argyrotaenia velutinana*, AF177640), BmorPBP (*Bombyx mori*, X94987), CfumPBP (*Choristoneura fumiferana*, AF177644), CrosPBP (*Choristoneura rosaceana*, AF177654), EposPBP1 (*Epiphyas postvittana*, AF416587), EposPBP2 (*E. postvittana*, AF411459), HarmPBP3 (*Helicoverpa armigera*, AF527054), HvirPBP (*Heliothis virescens*, X96861), HassPBP3 (*Helicoverpa assulta*, DQ286414), HzeaPBP (*Helicoverpa zea*, AF090191), LdisPBP1 (*Lymantria dispar*, AF007867), LdisPBP2 (*L. dispar*, AF007868), MbraPBP1 (*Mamestra brassicae*, AF051143), MbraPBP2 (*M. brassicae*, AF051142), MsexPBP1 (*Manduca sexta*, AF323972), MsexPBP2 (*M. sexta*, AF117588), MsexPBP3 (*M. sexta*, AF117581), OnubPBP (*Ostrinia nubilalis*, AF133631), PgosPBP (*Pectinophora gossypiella*, AF177656), SexiPBP (*Synanthedon exitiosa*, AF177657-AF177660), SnonPBP1 (*Sesamia nonagrioides*, AY485219), SnonPBP2 (*S. nonagrioides*, AY485220), and YcagPBP (*Yponomeuta cagnagellus*, AF177661). Conserved amino acids in all PBPs are shown in a *black background*. Positions of six conserved cysteine residues are indicated by *asterisks*

cDNA products of 275 and 281 bp, respectively. They were called SexigPBP1 and SexigPBP2, respectively. A RACE procedure was employed to obtain full-length sequences of PBP1 and PBP2 from *S. exigua*. Gene-specific primers (GSP) for 5′-RACE and 3′-RACE were derived from the sequences of the two cDNA products. The two PBP sequences of SexigPBP1 and SexigPBP2 (Figs. 1 and 2) were deposited in GenBank with the accession numbers AY540316 and AY545636, respectively.

The full-length sequences of *SexigPBP1* and *SexigPBP2* are 1,077 and 819 bp, respectively. *SexigPBP1* cDNA contains a 492-bp open reading frame that encodes a 164 amino acid protein, with a calculated molecular weight of 18.870 kDa and isoelectric point of 5.64. The initial 23 amino acids are predicted as a signal peptide, suggesting that the protein is secreted into the extracellular fluid. The mature SexigPBP1 comprises 141 amino acids, with a molecular weight of 16.413 kDa, and isoelectric point 5.34. *SexigPBP2* cDNA contains a 510-bp open reading frame that encodes a 170 amino acid protein, with

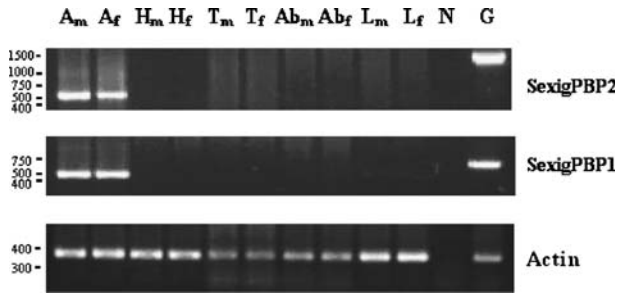


Fig. 4 Tissue-specific expression of *SexigPBP1* and *SexigPBP2*. RT-PCRs were performed using RNAs isolated from the different tissues. Amplification products were analyzed on agarose gels and visualized by UV illumination after ethidium bromide staining. Based on the primer design, the sizes of the expected PCR-products were 496 bp for *SexigPBP1*, 561 bp for *SexigPBP2*, and 377 bp for the control *Actin*. A_m male antenna, A_f female antenna, H_m male head (without antenna), H_f, female head (without antenna), T_m male thorax, T_f female thorax, Ab_m male abdomen, Ab_f female abdomen, L_m male leg, L_f, female leg, *N* no-template negative control ensures the specificity of the amplifications, *G* genomic DNA template reaction indicates the relative position of PCR product derived from genomic DNA contamination in experimental samples. The position of molecular weight markers (bp) is indicated on the *left side*

Table 2 Relative quantification data from *Spodoptera exigua* pheromone binding proteins (PBP) and β-actin amplified in separate wells

Gene	PBP C_T[a]	*ACTB* C_T[a]	ΔC_T[b]	$\Delta\Delta C_T$[c]	$2^{-\Delta\Delta C_T}$(range)[d]
Male PBP1	23.84±0.01	32.55±0.20	−8.71±0.21	0.00±0.21	1.00 (0.86–1.16)
Female PBP1	23.27±0.04	30.62±0.01	−7.35±0.05	1.36±0.05	0.39 (0.38–0.40)
Male PBP2	29.26±0.04	32.55±0.20	−3.29±0.23	0.00±0.23	1.00 (0.85–1.17)
Female PBP2	27.79±0.15	30.62±0.01	−2.83±0.15	0.46±0.15	0.73 (0.66–0.81)

[a] Mean±SE

[b] Mean PBP C_T−Mean *ACTB* C_T±SE

[c] Mean ΔC_T−Mean $\Delta C_{T,\ male}$±SE

[d] Normalized PBP amount relative to male antennae

calculated molecular weight of 18.881 kDa and isoelectric point 5.32. As the fragment of initial 27 amino acids is predicted as the signal peptide, the mature protein SexigPBP2 comprises 143 amino acids, has a calculated mass of 16.088 kDa, and an isoelectric point of 5.01. The two PBPs display similar hydropathy profiles (Supplementary material Fig. S1).

To ascertain the intron/exon structure of the genes, the two PBP genes were further cloned and sequenced from the genomic DNA preparation. Both *SexigPBP1* and *SexigPBP2* genes contain two introns and present a similar intron/exon structure. The two introns in *SexigPBP1* are located between Glu45 and Leu46 (intron1, 87 bp) and inside the codon for Asp105 (intron2, 142 bp). The introns in *SexigPBP2* are located between Glu49 and Met50 (intron1, 295 bp) and inside the codon for Asp110 (intron2, 644 bp). The ends of the introns have a typical GT-AG structure (Figs. 1 and 2).

We aligned the mature amino acid sequences deduced from *SexigPBP1* and *SexigPBP2* with the PBPs of other Lepidoptera (Fig. 3). Both SexigPBP1 and SexigPBP2 have conserved motifs of lepidopteran PBPs, including six cysteine residues forming three disulfide bridges and the position of the hydrophobic domains. The two PBPs from *S. exigua* share low similarity (44% identity), but are similar to PBPs of the same group from other insect species. SexigPBP1 displays a high identity with SnonPBP1 (72%), MbraPBP1 (71%), and AipsPBP2 (69%), whereas SexigPBP2 is more similar to HzeaPBP (86%), MbraPBP2 (85%), and HvirPBP (84%).

Tissue-specificity of PBP Gene Expression

To investigate the presence of PBPs in different tissues of *S. exigua*, RT-PCR experiments were performed with specific primers. Based on detailed genomic information, specific primer pairs were designed to span at least one intron region. This allowed us to distinguish amplification products resulting from cDNA templates from those resulting from possible genomic contamination of the cDNA preparation. Specific expression of the two PBPs in *S. exigua* was observed (Fig. 4). RT-PCR products of the predicted size were observed exclusively in reactions with antennal cDNA. No specific product was observed with head (without antennae), thorax, abdomen, or leg cDNA. The integrity of the cDNA templates prepared from different tissues was verified by primers specific for the actin gene. In all cDNA preparations, an *Actin* amplification product of the correct size was amplified. Larger bands that might have been generated from amplification of genomic DNA were not found. Therefore, *SexigPBP1* and *SexigPBP2* are only expressed in *S. exigua* antennae.

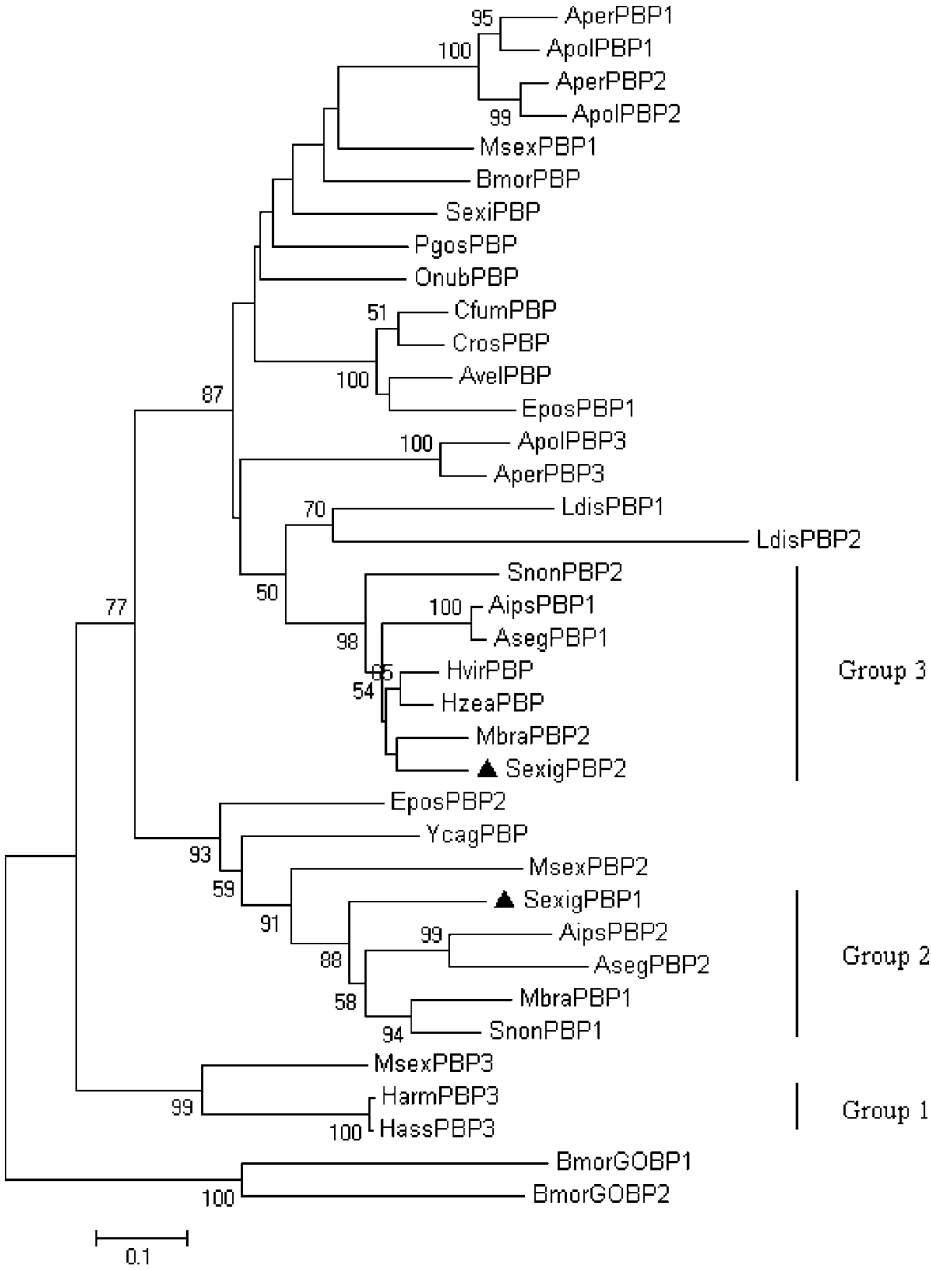


Fig. 5 Phylogenetic analysis based on SexigPBP1 and SexigPBP2 amino acid sequences. The tree was made with the neighbor-joining method using multiple alignments of amino acid sequences from Fig. 3. BmorGOBP1 and BmorGOBP2 from *Bombyx mori* are defined as the outgroup. Bootstrap analysis used 1,000 replications. Bootstrap values >50% are presented. SexigPBP1 and SexigPBP2 are marked with *arrows*. The *bar* indicates phylogenetic distance value

Comparison of PBP Gene Expression between Male and Female Antennae

Real-time PCR was performed to compare the transcript levels of *SexigPBP1* and *SexigPBP2* from antennae of male to female moths. For each sex, cDNA was synthesized from mRNA isolated from pools of 800 antennae. For sample analysis, the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to quantify the results obtained for *SexigPBP1* and *SexigPBP2* transcripts by real-time PCR. All samples were normalized with reference to *ACTB*. The absolute values of the slope of all lines from template dilution plots (log cDNA dilution vs. ΔC_T) were close to zero (data not shown). Therefore, the efficiencies of the target and reference genes were similar in our analysis, and the $\Delta\Delta C_T$ calculation method can be used for the relative quantification.

The transcription levels of PBP genes from antennae were higher in male moths than in females (Table 2). The amounts of *SexigPBP1* and *SexigPBP2* mRNA in female antennae were about 39 and 73%, respectively, relative to those in male antennae. It is noteworthy that the transcript level of *SexigPBP1* was much higher when compared to *SexigPBP2* transcript in both sexes. The transcription level of *SexigPBP1* was 50-fold higher than that of *SexigPBP2* in male moths, and *SexigPBP1* was 25-fold higher than *SexigPBP2* in female tissues.

Discussion

In this study, we identified two novel PBPs from antennae of *S. exigua*. The mature proteins contain 141 and 143 amino acids, respectively, and bear all the hallmarks of the PBP family including a signal peptide, a major hydrophobic domain, six conserved cysteine residues, and considerable similarities to other PBPs in amino acid sequence. The identification of the two SexigPBP genes will enable future studies of structure–function and evolutionary relationships of these genes with similar ones in related taxa.

Generally, high similarity in amino acid sequences means the same origin for the genes in question. In addition, if these genes share similar exon/intron structures in genomic DNA, they may have arisen from a shared ancestral gene by gene duplication. Both SexigPBP genes contain two introns with the same pattern reported for two *Agrotis* species, *A. segetum* and *A. ipsilon* (Picimbon and Gadenne 2002; Abraham et al. 2005). These conserved intron/extron structures and the intron positions, and the similar amino acid sequences of the PBPs (Fig. 3) suggest that the two PBP genes originated from duplication of a common PBP ancestral gene. However, the two introns differ in length within and between the PBP genes. The introns of *SexigPBP1* are much shorter than those of *SexigPBP2*, and intron1 is much shorter than intron2 in both PBP genes. It would be interesting to analyze the implication of these differences (particularly between PBP1 and PBP2) for PBP gene evolution and their functional diversification. However, more data are needed from intron/extron structures of PBPs from other insect species to facilitate such an analysis.

Real-time PCR indicated that *SexigPBP1* and *SexigPBP2* are expressed at different levels in both sexes, as is the case with other moths, such as *Bombyx mori* (L.) (Krieger et al. 1996; Maida et al. 2005), *M. brassicae* (Nagnan-Le Meillour et al. 1996), *H. zea* and *Spodoptera frugiperda* (Callahan et al. 2000), *A. ipsilon* (Picimbon and Gadenne 2002), and *Sesamia nonagrioides* (De Santis et al. 2006). The function and behavioral significance of PBPs in female moths still remains unclear. It is possible that female PBPs are involved in pheromone detection, as the autodetection of pheromonal compounds by females has been

demonstrated by electroantennography assay in *S. exigua* (our unpublished data) and in *Spodoptera littoralis* (Ljungberg et al. 1993; Ochieng et al. 1995), *Trichoplusia ni* (Seabrook et al. 1987), and *Helicoverpa armigera* (Fan et al. 2003).

Phylogenetic analysis showed that noctuid PBPs are classified into three distinct groups (Fig. 5), and SexigPBP1 and SexigPBP2 belong to two different groups, respectively. Group 1 contains HarmPBP3 and HassPBP3; group 2 includes SexigPBP1, AipsPBP2, AsegPBP2, MbraPBP1, and SnonPBP1; and group 3 contains SnonPBP2, AipsPBP1, AsegPBP1, HvirPBP, HzeaPBP, MbraPBP2, and SexigPBP2. This phylogenetic tree is somewhat different from that reported by Abraham et al. (2005), where HarmPBP3 and HassPBP3 were included in group 2 (group 1 in their nomenclature). In our analysis, however, HarmPBP3 and HassPBP3, and MsexPBP3 form a distinct group.

Our phylogenetic tree suggests that two duplication events might have occurred during noctuid PBP gene evolution. The first resulted in the divergence of group1/group 2–group 3, and the second gave rise to the divergence of group 2/group 3. Apart from the Noctuidae members, MsexPBP2 from Sphingidae, YcagPBP from Gelechiidae, and EposPBP2 from Tortricidae are closely related to group 2. Therefore, the duplication of group 2/group 3 might have occurred before the differentiation of these families. As for the duplication of group 1/group 2–group 3, it might have occurred even earlier. However, it is curious why PBPs of group 1 have been found just in three species, whereas those of groups 2 and 3 have been found in many species, especially in the Noctuidae. In our experiment with *S. exigua*, we successfully amplified the cDNAs for group 2 and 3 proteins, but using the same degenerate primers, we failed to obtain the cDNA for a hypothetical group 1 protein. We do not know the reasons for the failure to amplify the Group 1 protein cDNA for *S. exigua*. It is possible that PBP genes of group 1 may have been lost during later evolution in *S. exigua*. It is also possible that *S. exigua* possesses one or more group 1 protein PBP genes, but they remain to be cloned. A more meaningful phylogenetic tree of insect PBPs can be constructed when more PBPs become available from a larger number of species.

Acknowledgments We are grateful to professor Paolo Pelosi (University of Pisa) for his advice and useful comments on the manuscript. This work was supported by funds from the National Natural Science Foundation of China (grant number: 30571220) and Jiangsu Province Natural Science Foundation (grant number: BK2004098).

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J Chem Ecol (2007) 33:963–970
DOI 10.1007/s10886-007-9276-3

Biosynthesis of Tiglic, Ethacrylic, and 2-Methylbutyric Acids in a Carabid Beetle, *Pterostichus* (*Hypherpes*) *californicus*

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Received: 24 October 2006 / Accepted: 23 February 2007 /
Published online: 3 April 2007
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Abstract Tiglic, 2-methylbutyric, and ethacrylic acids are found in the pygidial gland defensive fluid of many carabid beetles. By injecting a deuterium-labeled precursor into the carabid beetle *Pterostichus* (*Hypherpes*) *californicus*, and analyzing the defensive fluid by gas chromatography/mass spectrometry, we were able to demonstrate that tiglic and ethacrylic acids are biosynthesized from isoleucine via 2-methylbutyric acid. Moreover, we observed that the injection of L-isoleucine induces an increased production of tiglic acid in *P. californicus*. A strong primary kinetic isotope effect was found to operate in the dehydrogenation step of 2-methylbutyric acid to tiglic and ethacrylic acids. Consequently, ethacrylic acid was found to preferentially accumulate the deuterium labeling from [2,3,4,4-$^{2}H_4$]isoleucine during our biosynthetic experiments.

Keywords Biosynthesis · Defensive allomones · Tiglic acid · 2-Methylbutyric acid · Ethacrylic acid · Isoleucine · Carabid beetles · *Pterostichus* (*Hypherpes*) *californicus* · Carabidae

Introduction

Carabid beetles are well known to produce a large variety of defensive allomones. Although information on the defensive chemistry of more than 500 species is now available (Will et al. 2000), little is known about the biosynthetic pathways by which these compounds are produced. Among the compounds characterized, small molecular weight carboxylic acids have been regarded as the most deterrent constituents against vertebrate or invertebrate predators. Tiglic acid is a frequently encountered allomone in the pygidial gland secretion

This paper is dedicated to Prof. Jerrold Meinwald on the occasion of his 80th birthday.

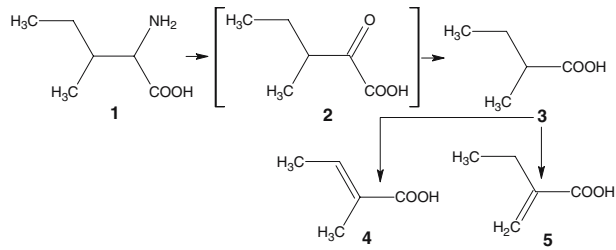
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of many carabid beetles. Tiglic acid derivatives are frequently encountered as plant secondary metabolites as well (Leete and Murrill 1967; Leete 1973). The biosynthesis of some tiglic derivatives in plants has been investigated, and isoleucine (**1**) has been shown to be the precursor of the acid moiety (Leete and Murrill 1967; Leete 1973). Presumably, 2-keto-3-methylvaleric acid (**2**) and 2-methylbutyric acid (**3**) are the key intermediates in this transformation, which can lead either to tiglic (**4**) or ethacrylic acid (**5**) (Scheme 1) (Leete 1973; Leete and Murrill 1967; Basey and Woolley 1973).

In beetles, the related biosynthetic origin of methacrylic acid from valine has been investigated (Benn et al. 1973; Attygalle et al. 1991). Benn et al. (1973) speculated that ethacrylic acid (**5**) might be produced from isoleucine in the pygidial gland in a manner similar to that of tiglic acid, perhaps without the intermediacy of tiglic or angelic acids (Scheme 1). However, molecular mechanisms leading to tiglic and ethacrylic acids in insects have not been elucidated, although Adachi et al. (1985) explored the biosynthetic origin of methacrylic and tiglic acids in *Carabus yaconinus* by using universally ^{14}C-labeled amino acids. From their results, L-valine and L-isoleucine were indicated to be the precursors of methacrylic acid and tiglic acid, respectively. However, results from universally labeled precursor experiments rarely provide molecular details of biosynthetic pathways. The availability of *Pterostichus* (*Hypherpes*) *californicus*, a beetle that produces tiglic acid, a minute amount of 2-methylbutyric acid, and traces of ethacrylic acid, gave us a chance to study the biosynthetic pathways of these five-carbon acids in detail.

Scheme 1 Biosynthesis of tiglic and ethacrylic acids



Methods and Materials

Live beetles were collected in the vicinity of Concord, California and transported to laboratory facilities in Berkeley, California. Beetles were kept in an environmental chamber simulating local day length and ambient temperatures and fed commercial dog food before and after injections. Before injection, beetles were agitated to cause them to spray their defensive compounds until no more perceptible liquid was expressed and the characteristic smell of volatile acids diminished. At this point, they were assumed to be nearly exhausted of stored defensive secretions. Beetles were then injected with ~2 µl of the treatment solution through the thin cuticle of the abdominal epipleura near tergum-VII. A solution of DL-[2,3,4,4-$^2H_4$]isoleucine/DL-*allo*-[2,3,4,4-$^2H_4$]isoleucine (Cambridge Isotope Laboratories, 5 mg/100 µl in insect saline) was injected into two adults. Untreated beetles, and those injected with L-isoleucine (Aldrich Chemical Company, 5 mg/100 µl in insect saline) were used as controls. After 2 d, beetles were frozen and the defensive glands were excised according to methods described by Will et al. (2000). Sealed glass ampoules containing excised gland reservoirs were shipped to laboratory facilities in Hoboken, New Jersey, for chemical analysis.

For chemical analysis, gland reservoirs were extracted with dichloromethane (about 20 μl). Extracts were concentrated, and nearly all the sample was injected to a gas chromatograph/mass spectrometer [a Shimadzu QP5050A mass spectrometer linked to a Shimadzu 17A gas chromatograph fitted with an FFAP-coated (0.25 μm film thickness) fused silica capillary column (30 m×0.25 mm)]. The oven temperature was held at 40°C for 3 min and increased at 6°C/min to 240°C. Electron-ionization (70 eV) mass spectra were recorded from *m/z* 35 to 300.

Results and Discussion

Gas chromatography/mass spectrometry analysis of volatile components of the pygidial gland extracts of *P.* (*Hypherpes*) *californicus* showed the presence of undecane, methacrylic acid, tiglic acid, acetic acid, propanoic acid, butanoic acid, isobutyric acid, 2-methylbutyric acid, 3-methybutyric acid, crotonic acid, senecioic acid, myristic acid, palmitic acid, stearic acid, oleic acid, and linoleic acid. Sections of the reconstructed chromatograms depicting peaks for compounds relevant to this biosynthetic investigation are shown in Fig. 1. All identifications were based on comparisons of mass spectra and retention times with those of the authentic samples. Peak 5 shown in Fig. 1b was a composite that represented traces of ethacrylic acid coeluting with crotonic acid.

Both 2-methylbutyric acid and 3-methylbutyric acid are present in the defensive secretion. Although these two acids coelute under the chromatographic conditions used (Fig. 1, peak 2), their mass spectra are distinguishably different, allowing unambiguous identification even when the spectrum recorded is a composite representing both

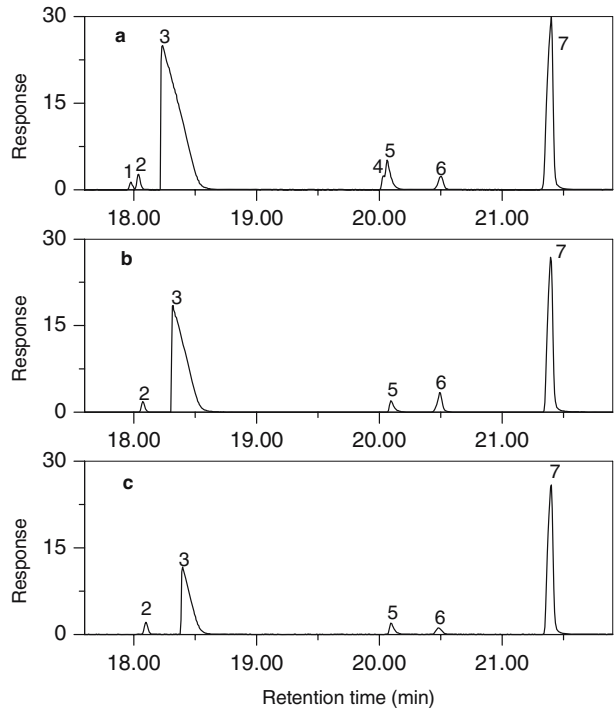


Fig. 1 Relevant sections of the reconstructed chromatograms obtained from the defensive fluid extracts of *P.* (*Hypherpes*) *californicus*: after DL-[2,3,4,4-$^2H_4$] isoleucine/DL-*allo*-[2,3,4,4-$^2H_4$] isoleucine was injected **(a)**; without injection **(b)**; and after L-isoleucine was injected **(c)**. Peak identifications: *1* 2-methyl [2,3,3-$^2H_3$]butyric acid, *2* 2-methylbutyric and 3-methylbutyric acids, *3* methacrylic acid, *4* 2-[1′,1′-$^2H_2$] ethacrylic acid, *5* crotonic acid with traces of ethacrylic acid, *6* solvent impurity with traces of senecioic acid, and *7* tiglic acid

compounds. For example, the mass spectrum of authentic 2-methylbutyric acid shows a peak for the ion *m/z* 74 (McLafferty rearrangement) as the base peak, and a minute peak at *m/z* 60 (Fig. 2b). In contrast, the spectrum of 3-methylbutyric acid shows its most predominant peak at *m/z* 60, and that at *m/z* 74 is negligible (Fig. 2c). Hence, from the peak intensities of *m/z* 60 and 74 observed in the composite mass spectrum (Fig. 2d) corresponding to chromatographic peak number 2 in Fig. 1b, recorded from pygidial gland extracts of untreated *P.* (*Hypherpes*) *californicus*, it was clear that both 2-methylbutyric acid and 3-methylbutyric acid are present in the secretion. This conclusion was further supported by gas chromatography/mass spectrometry analyses of authentic mixtures of 2-methylbutyric acid and 3-methylbutyric acid.

In the chromatogram recorded from glandular extracts of labeled-isoleucine-injected beetles, an additional chromatographic peak was observed immediately before that of 2-methylbutyric acid (peak 1, Fig. 1a). The mass spectrum corresponding to this chromatographic peak showed significant peaks at *m/z* 60, 75, and 90, although a molecular ion peak was absent (Fig. 2a). A comparison of this spectrum with that of authentic 2-methylbutyric acid (Fig. 1b) indicated that the spectrum represented 2-methyl

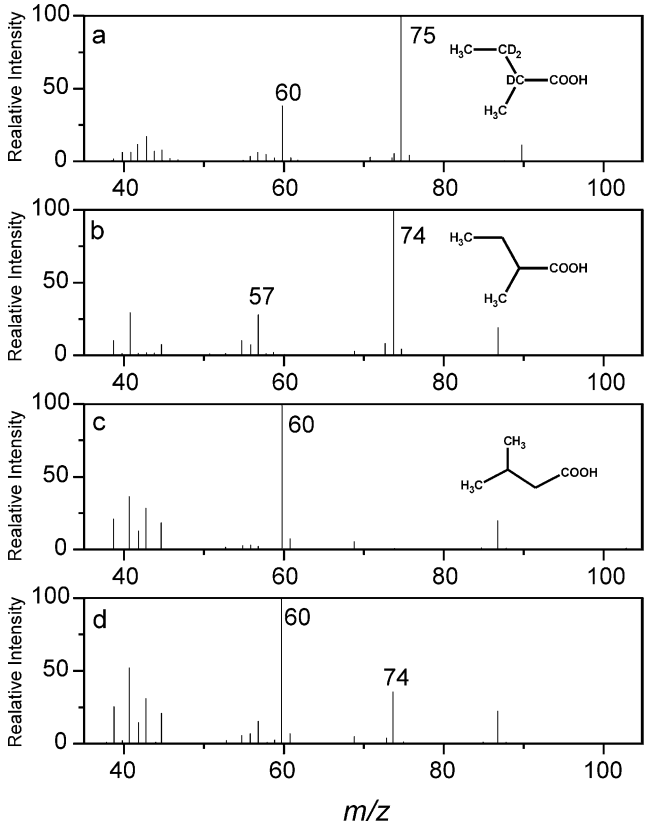
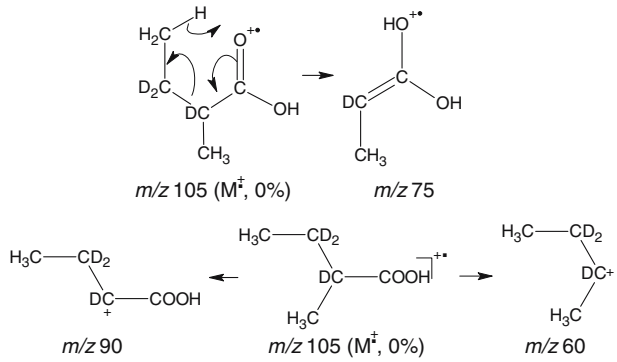


Fig. 2 Electron-ionization mass spectra corresponding to chromatographic peak 1 in Fig. 1a **(a)**, synthetic 2-methylbutyric acid **(b)**, synthetic 3-methylbutyric acid **(c)**, and that corresponding to chromatographic peak 2 of Fig. 1b **(d)**

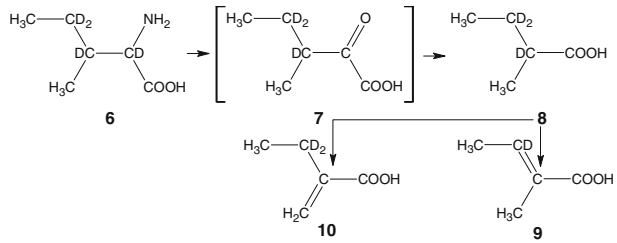
Scheme 2 Mass spectrometric fragmentation mechanisms proposed for 2-methyl [2,3,3-$^2H_3$]butyric acid



[2,3,3-$^2H_3$]butyric acid. Mass spectrometric fragmentation mechanisms, upon which this deduction was made, are illustrated in Scheme 2. A neutral molecule bearing two deuterons is lost during the McLafferty rearrangement to form the fragment ion observed at *m/z* 75. Deuteriated substances are known to elute before the nonlabeled parent compounds (Attygalle et al. 1991). Evidently, deuterium tagging from labeled-isoleucine has been incorporated specifically to 2-methylbutyric acid, and not to 3-methylbutyric acid. Hence, it was conclusive that 3-methylbutyric acid in *P.* (*Hypherpes*) *californicus* does not originate from a biosynthetic transformation of isoleucine. Evidently, [2,3,4,4-$^2H_4$]isoleucine (**6**) undergoes a deamination step, and the putative intermediate formed in this way, which is 2-keto-3-methyl[3,4,4-$^2H_3$]valeric acid (**7**), is converted by an oxidative decarboxylation step to 2-methyl[2,3,3-$^2H_3$]butyric acid (**8**) as postulated in Scheme 3.

The relative amount of tiglic acid present in glandular extracts of unlabeled L-isoleucine-injected beetles was significantly higher than that of untreated beetles (Fig. 1b, c). Apparently, the injection of L-isoleucine induces the production of tiglic acid. A careful mass chromatographic examination of acquired data corresponding to the chromatographic peak 7 in Fig. 1a, obtained from the glandular extracts of labeled-isoleucine-injected beetles, revealed that peak 7 represents both labeled and unlabeled tiglic acid. The ascending side of this chromatographic peak corresponded to a deuteriated tiglic acid bearing one deuterium atom (**9**, Scheme 3). When the background was subtracted from an average mass spectrum corresponding to the ascending side of the chromatographic peak, the resulting spectrum (Fig. 3a) unambiguously showed the labeled acid to be [3-$^2H_1$]tiglic acid (9), although the spectrum still had some contribution from the unlabeled tiglic acid (Fig. 3b). The mass spectral peaks observed at *m/z* 101, 86, and 56 indicated the presence of a deuteron in the labeled compound (Scheme 4, Fig. 3a). In all probability, a dehydrogenation step causes the biotransformation of 2-methylbutyric acid to tiglic acid.

Scheme 3 Biosynthetic transformation of [2,3,4,4-$^2H_4$]isoleucine



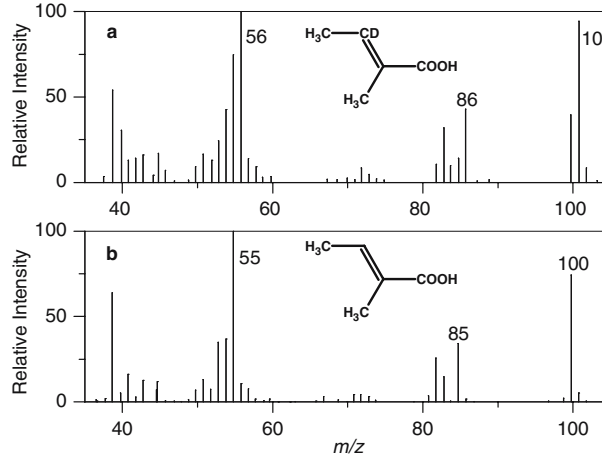
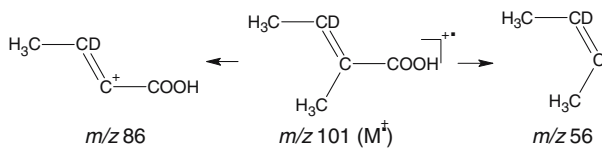


Fig. 3 Electron-ionization mass spectrum derived from the ascending side of chromatographic peak 7 of Fig. 1a (some contribution from unlabeled tiglic acid can still be observed) **(a)**, and that of synthetic tiglic acid **(b)**

Biosynthesis of Ethacrylic Acid (=2-Ethylacrylic Acid) (5) The chromatographic peak 5 in Fig. 1b is a composite peak that represents crotonic and traces of ethacrylic acids. In some other carabids, such as *Trachypachus gibbsii*, ethacrylic acid is the major constituent in its defensive secretion (Attygalle et al. 2004). The chromatogram recorded from samples obtained from labeling experiments with *P.* (*Hypherpes*) *californicus* showed a new chromatographic peak immediately before the composite peak for crotonic and ethacrylic acids (peak 4, Fig. 1a). The mass spectrum corresponding to this peak (Fig. 4a) was unambiguously that of 2-[1′,1′-$^2H_2$]ethacrylic acid (a.k.a 2-ethyl[1′,1′-$^2H_2$]acrylic acid, **10**) (Scheme 3). The fragment ions observed at *m/z* 102 and 57 indicated the presence of two deuterons in the labeled compound (Scheme 5, Fig. 4a). In other words, 2-methylbutyric acid also serves as the precursor of ethacrylic acid.

Our results indicate that depending on the dehydrogenation mechanism, the intermediate 2-methyl[2,3,3-$^2H_3$]butyric acid can eliminate either two deuterium atoms forming [3-$^2H_1$] tiglic acid, or one deuterium atom and a hydrogen atom forming 2-[1′,1′-$^2H_2$]ethacrylic acid. If the deuteriated ethacrylic acid were generated through the intermediacy of tiglic acid, then the nominal mass of labeled ethacrylic acid should have been 101. In this way, we were able to establish that both tiglic and ethacrylic acids are formed via 2-methylbutyric acid according to mechanisms suggested by Benn et al. (1973) over three decades ago (Scheme 1).

Relative Ratios of Labeled to Unlabeled Compounds An interesting observation was made when the intensity ratios of labeled to unlabeled compounds were estimated by integrating appropriate ion chromatograms. A comparison of chromatographic peak intensities of *m/z* 74 (for unlabeled 2-methylbutyric acid) and 75 (for labeled 2-methylbutyric acid), after relevant compensations were made for the natural isotopic abundances, revealed that about



Scheme 4 Rationalization of mass spectral peaks observed at *m/z* 101, 86, and 56 in the mass spectrum of [3-$^2H_1$]tiglic acid

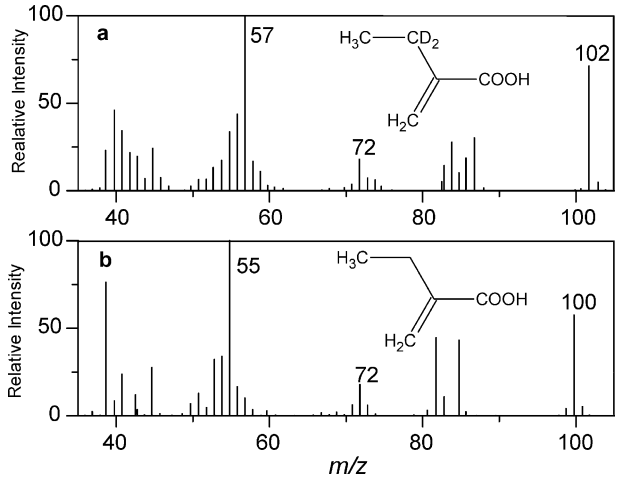
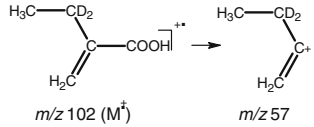


Fig. 4 Electron-ionization mass spectrum corresponding to chromatographic peak 4 of Fig. 1a **(a)**, and that of synthetic ethacrylic acid **(b)**

40% of the 2-methylbutyric acid had acquired the deuterium labeling under our experimental conditions. Similarly, the ion intensities of *m/z* 100 and 101 for unlabeled and labeled tiglic acid, respectively, showed a deuteriation of about 8%. Moreover, the relative amount of the labeled ethacrylic acid (*m/z* 102) was about seven times higher than that of the nonlabeled acid (*m/z* 100) [peaks 4 and 5, Fig. 1a (the major constituent in peak 5 is crotonic acid)]. Similar results were obtained from both beetles used in the labeling experiment. If 2-methybutyric, tiglic acid, and ethacrylic acid all came from the same isoleucine pool, how can these observations be rationalized? We suggest that a strong primary kinetic isotope effect operates in the dehydrogenation of 2-methylbutyric acid to tiglic and ethacrylic acids. For the formation of 2-[1′,1′-$^2H_2$]ethacrylic acid, an elimination of only one deuterium atom from 2-methyl[2,3,3-$^2H_3$]butyric acid is required. The production of [3-$^2H_1$]tiglic acid requires the removal of two deuterium atoms. As a consequence, the relative deuterium enrichment in the ethacrylic acid fraction compared to that in the tiglic acid fraction could be anticipated if 2-methylbutyric acid is the common precursor of these two unsaturated carboxylic acids. However, the unusually large incorporation of the deuterium label on ethacrylic acid cannot be rationalized entirely as a consequence of a kinetic isotope effect. Most likely, two different desaturases are involved in the dehydrogenation. At least to a certain extent, the preferential accumulation of the deuterium label on ethacrylic acid may be because of different enzyme activities and concentrations. No information is currently available on the nature of the desaturase enzymes.



Scheme 5 Formation of *m/z* 57 ion from 2-[1′,1′-$^2H_2$]ethacrylic acid

Acknowledgements The authors wish to thank Stevens Institute of Technology for financial support, and Aman Gill, UC, Berkeley, for assistance with handling and caring of beetle cultures. This research was funded (in part) by the United States Department of Agriculture, Cooperative State Research, Education, and Extension Service in partnership with the California State Agricultural Experiment Station.

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J Chem Ecol (2007) 33:971–984
DOI 10.1007/s10886-007-9274-5

Chemical Changes Associated with the Invasion of a *Melipona scutellaris* Colony by *Melipona rufiventris* Workers

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Received: 24 November 2006 / Accepted: 23 February 2007 /
Published online: 3 April 2007
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Abstract Wax constituents produced by worker bees and the chemistry of the nest batumen (mixture of wax, mud, and floral materials) in a *Melipona scutellaris* colony changed when it was invaded by *Melipona rufiventris* workers. Gas chromatography/mass spectrometry analyses showed that after invasion, the *M. scutellaris* workers of the invaded colony produced waxes with higher relative abundance of triacontanyl acetate and decreased the amounts of *n*-alkanes and *n*-9-alkenes. On the other hand, waxes from *M. rufiventris* workers displayed few changes. The change in the composition of the *M. scutellaris* waxes chemically differentiates that species from the *M. rufiventris* invader workers. Comparative analyses of batumens samples from pure and invaded colonies revealed greater amounts of terpenes and phenolic derivatives in the batumen from the invaded colony. This is the first report on the chemical characterization of batumens from stingless bees.

Keywords Meliponinae · *Melipona rufiventris* · *Melipona scutellaris* · Wax · Batumen · Triacontanyl acetate · Alkenes · Alkanes · Apidae

Introduction

Bees are among the world's most important pollinating agents. Their value in terms of the production of honey, wax, propolis, and other products used by humans is trivial when

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compared to their central role in pollination (Michener 2000). Stingless bees are responsible for pollinating 40 to 90% of the native flora in tropical regions where they occur, including Brazil, and play a fundamental role in perpetuating ecosystems (Nascimento et al. 2000). The destruction of forests and savannas has threatened the survival of a large number of bee species; thus, preservation of wild bee species is of paramount importance to biodiversity conservation and one of the reasons for the present study.

Among the corbiculate Apidae (Euglossinae, Bombinae, Meliponinae, and the Apinae), only two major groups of highly social bees are found: the stingless honeybees of the tropics and the true honeybees, the Meliponinae and the Apinae, respectively (Winston and Michener 1977). The social organization of the Meliponinae requires complex social interactions inside their nests, as well as in the surrounding environment. Nest construction is of major importance in the integration of the colony, and both the provisions and brood contained in the nest have profound influences on the behavior and caste determination of the bees (Roubik 1989). To construct their nests, the Meliponinae use cerumen, which is a mixture of wax and floral resins (propolis) gathered in large amounts by the forager bees. Cerumen is utilized in different ratios in storage pots, brood cells, entrance openings and tubes, pillars, involucrum layers, and in royal cameras to imprison gynes. The nests of stingless bees are surrounded and protected by a structure called batumen, a wall-like plate made up of a brittle cerumen produced by mixing wax with floral oils and floral resins (propolis), to which is added mud, sometimes even pieces of flowers, and leaves (Roubik 1989; Nogueira-Neto 1997). When excessive mud is added, the mixture is called "geopropolis" (Nogueira-Neto 1997), and this is employed in the peculiar design of the nest entrance and in the batumen of many *Melipona* species, including *Melipona scutellaris* and *Melipona rufiventris*. Pure propolis, pure wax, and cerumen are stored in the nest and used in construction and defense (Roubik 1989).

The ability to recognize nestmates and distinguish them from individuals of other colonies is a key property of social insects (Obin 1986; Gamboa et al. 1991; Billen 2006). After brief antennation, social wasps, bees, and ants typically show an immediate discriminative response toward nestmates compared to others that are not members of the colony. The profiles of cuticular substances, particularly hydrocarbons, are often species- and colony-specific, and the quality of interactions among individuals is correlated with similarities or differences in the hydrocarbon profiles, indicating that cuticular hydrocarbons play a role in nestmate recognition and even reproduction (Bonavita-Cougourdan et al. 1987; Morel and Blum 1988; Nowbahari et al. 1990; Singer 1998; Simmons et al. 2003; Beekman 2004).

Perception of Apidae to substances such as floral odors, bee pheromones, and chemicals is sensitive. Thus, bee behavioral responses can be determined by inter- and intraspecific olfactory stimuli, and a good example is provided by the cleptobiotic bee *Lestrimellita limao*, which uses citral to disrupt the organization inside the *M. rufiventris* colony (Pompeu and Silveira 2005). In *Melipona*, as in other Apidae, wax, mandibular, and Dufour glandular secretions are responsible for intraspecific chemical communication. Glandular secretions determine caste and sexual hierarchy, following a rather specific pattern in each species (Cruz-López et al. 2001, 2002; Patricio et al. 2003).

The temporary host–parasitic interaction between *M. rufiventris* and *M. scutellaris* (Apidae: Meliponinae) is an uncommon phenomenon, where *M. rufiventris* workers invade a *M. scutellaris* colony. Roubik was the first to observe this (Nogueira-Neto 1997) and hypothesized that the invasion can be understood by nest guarding, which is based on the perception of the characteristic scent of each species. At the nest entrance, bees assess the scents of other bees, and a guard bee will not let an individual pass if it lacks the correct smell. This hypothesis, however, has not been proven.

To address whether bees alter their chemistry (waxes) and colony environment (nests) upon the presence of an invader species, two aspects of a *M. scutellaris* colony invaded by *M. rufiventris* workers were studied: (1) the chemical compositions of abdominal waxes of bee workers, and (2) the chemical constituents of the nest batumen, which is composed of bee waxes and external material collected by worker bees, thus reflecting the global activity of the colony. Wax and batumen samples were gathered from pure colonies of each species, and the chemistry was compared to samples collected from a *M. scutellaris* colony invaded by *M. rufiventris* workers.

Methods and Materials

Biological Material Abdominal waxes from worker bees of *M. rufiventris* and *M. scutellaris* and nest batumens were collected at Aretuzina farm in São Simão, São Paulo, Brazil, where colonies of both species have been established for many years. Waxes were collected from worker bees living in three different colonies: colony 1, a pure colony of *M. scutellaris* (N=4), colony 2, a pure colony of *M. rufiventris* (N=4), and colony 3, a *M. scutellaris* colony invaded by *M. rufiventris* (N=4 for each species).

Batumen samples (N=2) were collected from each of the three colonies above.

Reagents *n*-Alkane standards (C_{10}–C_{37}) were obtained from petroleum (100 ml), by silica gel column chromatography using bidistilled hexane as solvent. The distilled and bidistilled solvents (Synth, São Paulo, Brazil) were obtained according to the method described by Perrin et al. (1980). Iodine (Rothyl, New York, USA), dimethyl disulfide (DMDS) (Aldrich, Steinheim, Germany), magnesium sulfate (Nuclear, Brazil), and sodium thiosulfate (Merck, Rio de Janeiro, Brazil) were used.

Wax Sample Preparations Abdomens of *M. rufiventris* (N=4) and *M. scutellaris* (N=4) workers were washed with bidistilled hexane (2 ml). The solvent was evaporated under a nitrogen stream to produce around 0.4 mg of crude extract in the form of a white wax. The wax samples were dissolved in 0.5 ml of ethyl acetate, and analyzed by gas chromatography/mass spectrometry (GC-MS) by injecting 1 μl quantities (Table 1).

Batumen Chromatographic Fractionation Equal amounts of batumens samples (805 mg) from pure colonies and the invaded colony were collected, and their constituents were separated by silica gel 60 (0.035–0.070 mm, Merck, Dannstadt, Germany, 20 g) column chromatography (h=23 cm, id=2.5 cm), eluted with solvents of increasing polarity (500 ml to each fraction) (Table 2). Solvent evaporation furnished five fractions for each batumen: A (hexane), B (hexane/dichloromethane 1:1), C (dichloromethane), D (dichloromethane/ethyl acetate 1:1), and E (ethyl acetate). Each fraction was analyzed by GC-MS by injecting 1 μl (2 mg in 0.5 ml of ethyl acetate) (Table 3). Polar compounds and dirt from batumens remained on the top of the silica column and were not analyzed.

GC-MS Analyses Analyses were carried out by using an HP 6890/5973 system equipped with an HP5 fused silica capillary column (30 m×0.25 mm×0.25 μm). Helium was used as carrier gas at a flow rate of 1 ml/min, splitless mode. The MS were taken at 70 eV, and the scanning speed was 2.89 scans/sec from *m/z* 40 to 550. The interface temperature was maintained at 280°C. Injector temperature was 240°C for the extracts of worker bees and 250°C

Table 1 Relative abundances of constituents in abdominal waxes obtained from *M. scutellaris* and *M. rufiventris* workers in pure and invaded colonies

Peak Number	Compounds	t_R (min)	RI (calc.)	RI (lit.)	*M. scutellaris* pure colony (%)[a]	*M. scutellaris* invaded colony (%)[a]	*M. rufiventris* pure colony (%)[a]	*M. rufiventris* invaded colony (%)[a]
1	9-Tricosene (isomer 1)	32.70	2206	–	–	–	0.04	–
2	9-Tricosene (isomer 2)	32.80	2276	–	–	–	0.01	–
3	Tricosane	32.90	2300	2300	0.12	–	2.25	3.49
4	Tetracosane	33.28	2400	2400	–	–	0.29	2.31
5	*E*-9-Pentacosene	34.17	2467	–	0.67	–	0.12	0.01
6	*Z*-9-Pentacosene	34.28	2476	–	0.01	–	0.01	–
7	Pentacosane	34.60	2500	2500	1.64	0.67	16.65	10.92
8	*Z*-9-Hexacosene	35.60	2577	–	0.01	–	–	–
9	Hexacosane	35.90	2600	–	0.14	–	0.96	2.45
10	*E*-9-Heptacosene	36.82	2671	–	9.78	1.30	0.97	2.67
11	*Z*-9-Heptacosene	36.90	2677	–	0.01	–	0.14	0.01
12	Heptacosane	37.20	2700	–	8.39	1.57	19.21	10.61
13	*Z*-9-Octacosene	38.14	2777	–	–	–	0.16	0.01
14	Octacosane	38.42	2800	–	0.29	–	0.60	2.33
15	Nonacosadiene[b]	38.99	2848	–	0.01	–	0.42	0.01
16	*E*-9-Nonacosene	39.20	2865	–	3.63	0.01	0.56	7.87
17	*Z*-9-Nonacosene	39.35	2878	–	0.19	–	9.21	2.37
18	Nonacosane	39.62	2900	–	8.28	0.99	7.08	4.19
19	9-Triacontene (isomer 1)	40.31	2966	–	0.29	–	0.04	2.30
20	9-Triacontene (isomer 2)	40.42	2977	–	–	–	0.35	–
21	Triacontane	40.66	3000	–	0.01	–	0.13	–
22	Hentriacontadiene (isomer 1)[b]	41.20	3049	–	–	–	1.50	2.40
23	Hentriacontadiene (isomer 2)[b]	41.26	3054	–	–	–	0.89	2.44
24	Hentriacontadiene (isomer 3)[b]	41.32	3059	–	–	–	0.41	0.01
25	*E*-9-Hentriacontene	41.36	3063	–	19.89	2.34	3.19	2.77
26	*Z*-9-Hentriacontene	41.55	3080	–	–	–	14.20	11.36
27	Hentriacontane	41.77	3100	–	3.81	0.01	2.27	2.57
28	9-Dotriacontene (isomer 1)	42.36	3167	–	0.27	–	0.09	2.31
29	9-Dotriacontene (isomer 2)	42.50	3183	–	–	–	0.29	–
30	Dotriacontane	42.65	3200	–	0.01	–	–	–
31	Tritriacontadiene (isomer 1)[b]	43.29	3255	–	–	–	2.81	3.13
32	Tritriacontadiene (isomer 2)[b]	43.36	3262	–	–	–	0.83	–
33	9-Tritriacontene (isomer 1)	43.49	3273	–	6.43	0.55	1.88	2.92
34	9-Tritriacontene (isomer 2)	43.64	3286	–	–	–	3.99	3.81

Table 1 (continued)

Peak Number	Compounds	t_R (min)	RI (calc.)	RI (lit.)	*M. scutellaris* pure colony (%)[a]	*M. scutellaris* invaded colony (%)[a]	*M. rufiventris* pure colony (%)[a]	*M. rufiventris* invaded colony (%)[a]
35	Tritriacontane	43.81	3300	–	2.05	–	0.28	2.31
36	Triacontanyl acetate	45.06	3413	–	21.54	82.27	–	–
37	9-Pentatriacontene (isomer 1)	45.65	3466	–	0.76	–	0.11	–
38	9-Pentatriacontene (isomer 2)	45.85	3484	–	–	–	0.09	–
39	Pentatriacontane	46.03	3500	–	0.25	–	–	–
40	Octadecyl hexadecanoate	46.62	3553	–	0.17	–	–	–
41	Octadecenyl octadecenoate	48.40	3714	–	0.31	–	–	–
42	Octadecyl octadecenoate	48.60	3732	–	0.78	–	–	–
–	Unidentified	–	–	–	10.26	10.29	7.97	12.42
TOTAL					100.00	100.00	100.00	100.00

There are N=4 workers for each sample. The compounds are listed in the order of their retention times.

t_R Retention times, *RI (calc.)* calculated retention indexes, *RI (lit.)* retention indexes taken from literature (Adams 1995), – not found

[a] Relative abundance (%)

[b] The double bond positions in alkadienes were not determined.

for the batumen fractions. The oven temperature program varied with the samples: for wax samples, from 50 to 310°C at 6°C/min with a final hold time of 15 min; for batumen fraction A, from 50 to 290°C at 12°C/min with a final hold time of 10 min; for batumen fractions B, C, and E, from 50 to 290°C at 12°C/min with a final hold time of 20 min; for batumen

Table 2 Chemical fractions of batumen samples obtained from (1) pure colony of *M. scutellaris*, (2) pure colony of *M. rufiventris*, and (3) invaded colony of *M. scutellaris*

Fractions	Solvents	Batumen 1 (mg)	Batumen 2 (mg)	Batumen 3 (mg)
A	Hexane	2	2	3
B	Hexane/dichloromethane (1:1)	6	5	35
C	Dichloromethane	5	4	11
D	Dichloromethane/ethyl acetate (1:1)	6	3	28
E	Ethyl acetate	6	2	22
–	Were not analyzed[a]	780	789	706
TOTAL		805	805	805

[a] Polar compounds and, principally, mud are the major part from batumens of these species.

Table 3 Identified compounds of the batumen fractions from (1) pure colony of *M. scutellaris*, (2) pure colony of *M. rufiventris*, and (3) invaded colony of *M. scutellaris*

Compounds	t_R (min)	RI (calc.)	RI (lit.)	Batumen 1 (%)[a]	Batumen 2 (%)[a]	Batumen 3 (%)[a]
Fraction A						
3-Methyl-tridecane	9.56	1371	–	–	–	0.35
Tetradecane	9.89	1400	1399	9.31	3.63	3.41
β-Caryophyllene	10.12	1421	1418	–	–	0.51
(α, *trans*)-Bergamotene	10.28	1436	1436	–	–	3.16
α-Guaiene	10.34	1442	1439	–	–	0.24
γ-Gurjunene	10.66	1472	1473	–	–	1.35
(*cis*, β)-Guaiene	10.82	1487	1490	–	–	0.76
Pentadecane	10.96	1500	1500	0.28	0.01	–
δ-Guaiene	11.02	1506	1505	–	–	2.81
γ-Cadinene	11.09	1513	1513	–	–	1.71
γ-Bisabolene	11.11	1515	1515	0.40	–	–
(1*S*, *cis*)-Calamenene	11.17	1521	1521	–	–	0.88
Methyl dodecanoate	11.23	1523	1525	0.37	–	–
δ-Cadinene	11.26	1528	1524	0.85	2.73	2.03
4-Ethyl-tetradecane	11.41	1544	–	0.70	2.83	0.59
3-Methyl-pentadecane	11.68	1571	–	2.86	5.01	2.20
Hexadecene[b]	11.90	1592	–	0.60	2.97	0.46
Hexadecane	11.98	1600	1600	8.69	9.26	5.98
4-Methyl-hexadecane	12.59	1664	–	0.71	2.88	0.45
Heptadecane	12.93	1700	1700	0.50	–	0.19
3-Methyl-heptadecane	13.59	1772	–	2.51	–	1.68
Octadecene[b]	13.79	1793	–	0.28	2.67	0.17
Octadecane	13.85	1800	1800	2.52	3.87	1.57
Nonadecane	14.72	1900	1900	–	2.99	–
3-Methyl-nonadecane	15.31	1971	–	0.70	2.78	0.43
Eicosane	15.55	2000	2000	0.62	2.94	–
Abietadiene	16.22	2085	2080	0.68	0.01	1.94
9-Tricosene	17.65	2276	–	0.17	–	0.17
Tricosane	17.82	2300	2300	0.29	2.86	0.26
Z-9-Pentacosene	19.03	2476	–	1.41	–	1.82
Pentacosane	19.19	2500	2500	1.94	4.19	1.57
Z-9-Hexacosene	19.69	2577	–	–	–	0.46
Hexacosane	19.84	2600	–	0.22	0.01	0.18
Z-9-Heptacosene	20.34	2677	–	9.79	2.94	11.49
Heptacosane	20.49	2700	–	7.17	4.22	5.38
Octacosane	21.21	2800	–	0.27	–	0.24
Squalene	21.34	2815	–	3.17	3.34	1.57
Z-9-Nonacosene	21.86	2877	–	2.99	5.37	2.85
Nonacosane	22.05	2900	–	6.10	3.63	4.55
Z-9-Hentriacontene	23.96	3079	–	14.62	5.63	12.75
Hentriacontane	24.21	3100	–	2.31	3.31	2.73
9-Tritriacontene	27.00	3279	–	4.76	–	5.79
Tritriacontane	27.36	3300	–	1.28	–	1.81
Unidentified	–	–	–	10.93	19.92	13.51
TOTAL				100.00	100.00	100.00
Fraction B						
Tetradecane	9.89	1400	1399	0.63	4.15	–

Table 3 (continued)

Compounds	t_R (min)	RI (calc.)	RI (lit.)	Batumen 1 (%)[a]	Batumen 2 (%)[a]	Batumen 3 (%)[a]
(α, *trans*)-Bergamotene	10.28	1436	1436	–	–	0.14
2,6-di-*tert*-Butylquinone	10.65	1471	–	0.20	2.98	–
Pentadecane	10.96	1500	1500	0.60	3.99	–
Hexadecane	11.98	1600	1600	0.25	3.22	–
Caryophyllene oxide	12.00	1602	1581	–	–	0.37
Heptadecane	12.93	1700	1700	0.22	2.89	–
Octadecane	13.85	1800	1800	0.12	3.05	–
Eicosane	15.55	2000	2000	0.15	3.20	–
Manoyl oxide	15.91	2046	2010	–	2.94	–
Heneicosane	16.34	2100	2100	0.22	3.37	–
Docosane	17.10	2200	2200	0.24	3.53	–
Tetracosane	18.52	2400	2400	0.29	3.44	–
Pentacosane	19.19	2500	2500	0.36	3.76	–
Hexacosane	19.84	2600	–	–	3.30	–
Z-9-Heptacosene	20.34	2677	–	0.30	–	–
Heptacosane	20.49	2700	–	0.36	3.43	–
Octacosane	21.21	2800	–	–	3.07	–
Squalene	21.34	2816	–	0.20	–	–
Z-9-Nonacosene	21.86	2878	–	–	3.21	–
Nonacosane	22.04	2900	–	0.32	3.07	–
bis (Octylphenyl)-amine	22.71	2968	–	2.87	8.74	–
β-Amyrene	28.01	3330	–	0.75	4.48	1.23
Lanosterol	28.28	3343	–	–	–	1.63
β-Amyrine	28.61	3358	–	8.80	–	12.04
3-keto-Ursene	28.91	3372	–	4.45	10.82	5.77
Cycloart-24-en-3β-ol	29.43	3396	–	34.28	–	41.05
α-Amyrine	29.61	3404	–	14.59	–	13.14
Triacontanyl acetate	29.86	3413	–	12.60	–	5.69
24-Methylene-cycloartanol	30.68	3444	–	4.04	–	10.22
Unidentified	–	–	–	13.16	19.36	8.72
TOTAL				100.00	100.00	100.00
Fraction C						
4-*tert*-Butylphenol	8.69	1294	–	–	–	1.06
Tridecane	8.76	1300	1299	–	1.66	–
Tetradecane	9.89	1400	1399	9.07	8.44	–
Pentadecane	10.96	1500	1500	8.22	6.60	–
Hexadecane	11.97	1600	1600	3.71	3.00	–
Heptadecane	12.93	1700	1700	3.84	2.89	–
Octadecane	13.85	1800	1800	2.89	2.05	–
Nonadecane	14.72	1900	1900	2.73	1.98	–
Eicosane	15.54	2000	2000	3.34	2.38	–
Heneicosane	16.34	2100	2100	3.32	2.63	–
Docosane	17.10	2200	2200	3.78	3.35	–
Unknown triterpene	17.99	2324	–	–	–	24.87
Tetracosane	18.52	2400	2400	4.16	3.32	–
Junipene	18.55	2404	–	–	–	8.75
Pentacosane	19.19	2500	2500	4.31	3.57	–
Hexacosane	19.84	2600	–	3.89	3.09	7.53
Heptacosane	20.48	2700	–	3.69	3.16	–

Table 3 (continued)

Compounds	t_R (min)	RI (calc.)	RI (lit.)	Batumen 1 (%)[a]	Batumen 2 (%)[a]	Batumen 3 (%)[a]
Octacosane	21.21	2800	–	–	2.45	–
Nonacosane	22.04	2900	–	–	2.36	–
bis (Octylphenyl)-amine	22.71	2968	–	13.87	21.31	9.72
Dammaradienol	34.02	3557	–	–	–	12.12
Unidentified	–	–	–	29.18	25.76	29.47
TOTAL				100.00	100.00	100.00
Fraction D						
Toluene 2,4-diisocyanate	9.46	1362	–	–	–	0.38
Tetradecane	9.86	1400	1399	3.96	5.43	–
N,*N*-Dicyclohexylamine	10.22	1431	1411	–	3.74	–
Pentadecane	10.96	1500	1500	3.04	4.32	–
Hexadecane	11.97	1600	1600	–	1.62	–
Heptadecane	12.93	1700	1700	–	1.59	–
Octadecane	13.85	1800	1800	–	1.23	–
Metetilachlor	15.38	1980	–	2.62	1.38	0.98
Eicosane	15.54	2000	2000	–	1.49	–
Heneicosane	16.34	2100	2100	–	1.52	–
p-Isopropylaminodiphenylamine	16.56	2129	–	8.08	3.61	–
Docosane	17.10	2200	2200	2.14	1.78	–
tris (1,3-Dichlor-2-propyl) phosphate	18.03	2330	–	–	–	0.41
Tetracosane	18.52	2400	2400	2.44	2.09	–
Pentacosane	19.19	2500	2500	–	2.25	–
Hexacosane	19.84	2600	–	2.38	2.02	–
Heptacosane	20.48	2700	–	–	2.66	–
Oleamide	21.10	2785	–	8.21	11.25	1.05
Octacosane	21.21	2800	–	–	2.13	–
Resorcinol, 5-pentadecyl	21.34	2816	–	–	–	3.28
bis (Octylphenyl)-amine	22.72	2969	–	9.89	13.66	–
Resorcinol, 5-heptadecadienyl	22.96	2993	–	–	–	7.72
Resorcinol, 5-heptadecenyl	23.14	3009	–	–	–	29.74
Resorcinol, 5-nonadecenyl	25.98	3220	–	–	–	14.08
Epiendesmin	26.42	3245	–	30.55	1.44	3.84
Unidentified	–	–	–	26.69	34.79	38.52
TOTAL				100.00	100.00	100.00
Fraction E						
2,3-Dihydro-benzofuran	7.84	1223	–	0.70	0.93	–
N,*N*-Dicyclohexylamine	10.22	1411	1411	0.51	19.14	–
Dodecanoic acid	11.55	1558	1568	–	1.91	–
Hexadecanoic acid	15.20	1959	–	0.58	2.97	–
Octadecenoic acid	16.62	2137	–	–	2.11	–
Octadecanoic acid	16.80	2161	–	–	0.51	–
Phenol, 3-pentadecyl	19.22	2505	–	–	–	1.09
Phenol, 3-heptadecadienyl	20.39	2685	–	–	–	3.75
Phenol, 3-heptadecenyl	20.51	2704	–	–	–	10.15
Phenol, 3-heptadecyl	20.59	2715	–	–	–	7.47
Oleamide	21.10	2785	–	–	7.25	–
Phenol, 3-nonadecenyl	22.09	2905	–	–	–	4.53
Phenol, 3-nonadecyl	22.27	2923	–	–	–	3.70

Table 3 (continued)

Compounds	t_R (min)	RI (calc.)	RI (lit.)	Batumen 1 (%)[a]	Batumen 2 (%)[a]	Batumen 3 (%)[a]
bis (Octylphenyl)-amine	22.72	2969	–	–	1.61	–
Epiendesmin	26.42	3245	–	7.57	7.14	1.02
Unidentified	–	–	–	90.64	56.43	68.29
TOTAL				100.00	100.00	100.00

t_R Retention times, *RI (calc.)* calculated retention indexes, *RI (lit.)* retention indexes taken from literature (Adams 1995), – not found

[a] Relative abundance (%)

[b] The double bond position was not determined for this alkenes.

fraction D, from 50 to 290°C at 12°C/min with a final hold time of 22 min; and for derivatized samples, from 50 to 290°C at 12°C/min with a final hold time of 50 min.

Retention Indexes Wax samples and fractions of the batumen were coinjected with a hydrocarbon mixture. Retention indexes (RI) were calculated according to the Van den Dool and Kratz equation (Van den Dool and Kratz 1963) and compared with the literature (Adams 1995). Retention indexes for the *n*-alkane standards (C_{10}–C_{37}) were taken as whole numbers from 1,000 to 3,700.

The *Z* and *E* alkenes (C_{25}–C_{31}) were identified by applying the equivalent chain length (ECL), which is 100 times smaller than the retention indexes (ECL=RI/100) (Ashes et al. 1980), and by comparison with literature values (Howard and Baker 2003).

Derivatizations The position of the double bonds in alkenes present in waxes and batumens samples was determined by derivatization with DMDS. The samples (~1 mg) were dissolved in bidistilled hexane (2 ml) and treated with DMDS (200 µl) and 100 µl of iodine solution (32 mg of I_2 in 2 ml of distilled diethyl ether). Reaction mixtures were stirred overnight at 50°C, and quenched with 2 ml of aqueous sodium thiosulfate solution (1 g of $Na_2S_2O_3$ in 10 ml of distilled water). The organic phase was extracted, dried over anhydrous magnesium sulfate, and evaporated to dryness under a nitrogen stream (Buser et al. 1983; Vincenti et al. 1987). The derivatized samples were dissolved in bidistilled hexane and analyzed by GC-MS.

Identification of Chemical Compounds Compounds in wax and batumen were identified by comparing their mass spectra with those in the equipment library (Wiley, 275). Pure substances were further analyzed by NMR.

NMR Analyses The ^{1}H NMR spectrum was recorded with a Varian 300 MHz spectrometer, using $CDCl_3$ as solvent and TMS as internal standard. The residual solvent ($CDCl_3$) signal at δ 77.00 was used as internal reference in ^{13}C NMR analyses at 75.5 MHz. Methyl, methylene, and quaternary carbons were discriminated by using distortionless enhancement by polarization transfer (DEPT) 135° and 90°.

Results

Pure Colonies Gas chromatography/mass spectrometry analyses of abdominal waxes from *M. scutellaris* (Fig. 1a, b) and *M. rufiventris* workers (Fig. 2a, b) living in pure and invaded

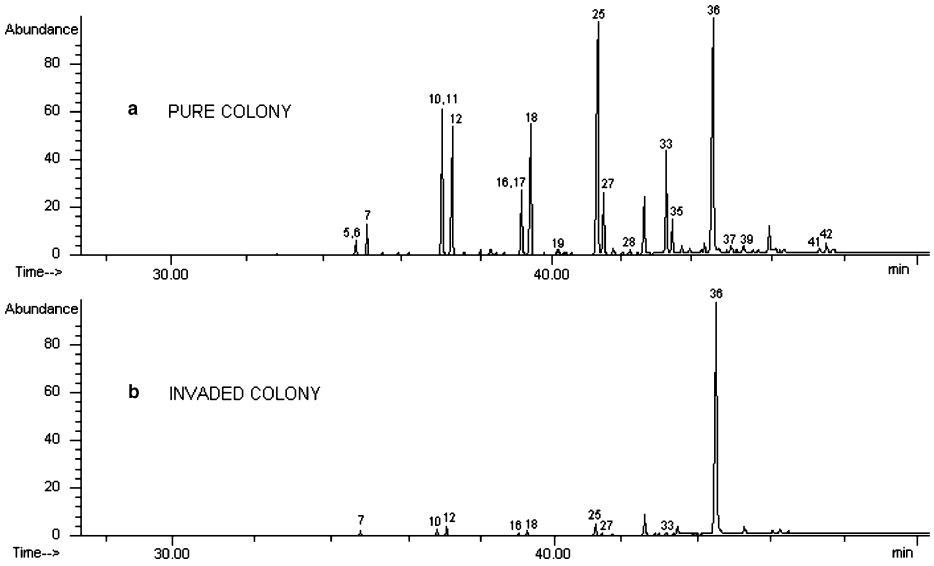


Fig. 1 Total ion current chromatograms of *M. scutellaris* workers' abdominal waxes: **a** pure colony and **b** invaded colony

colonies revealed several substances (Table 1). Wax from *M. scutellaris* workers living in a pure colony was mainly composed of *n*-alkanes (24.99%), *n*-9-alkenes (41.94%), and triacontanyl acetate (21.54%) (Fig. 1a, Table 1). Wax from *M. rufiventris* workers living in a pure colony was mainly composed of *n*-alkanes (49.72%), *n*-9-alkenes (35.45%), and alkadienes (6.86%) (Fig. 2a, Table 1).

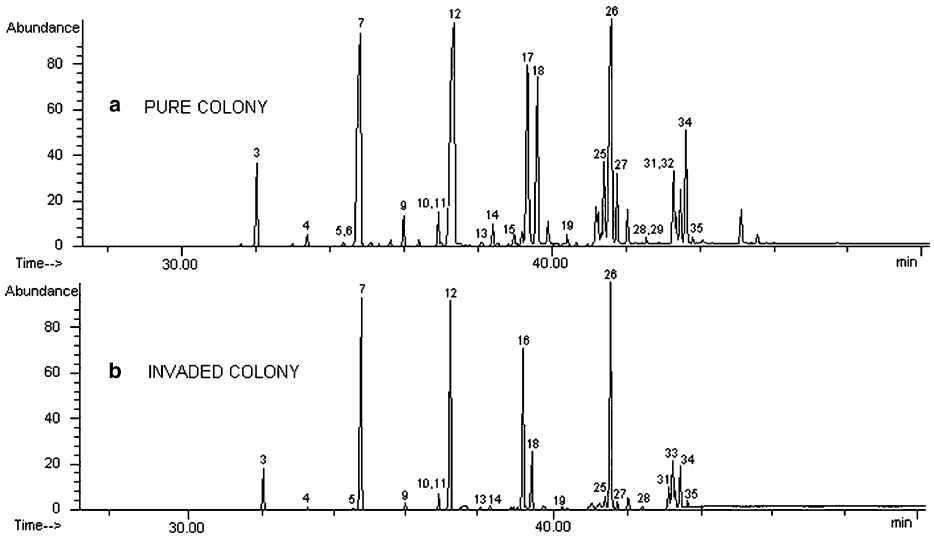


Fig. 2 Total ion current chromatograms of *M. rufiventris* workers' abdominal waxes: **a** pure colony and **b** invaded colony

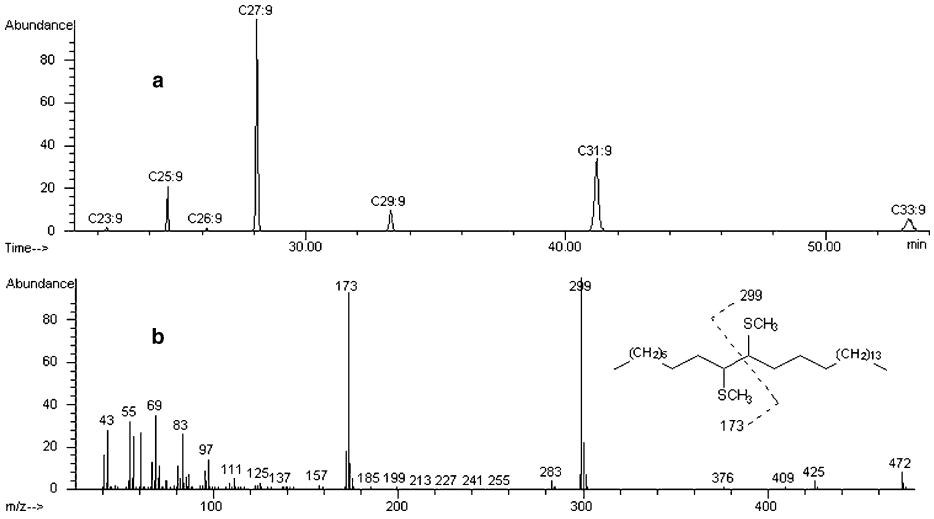


Fig. 3 **a** Ion extraction chromatogram (*m/z* 173) of DMDS derivatives of *n*-9-alkenes from invaded colony batumen and **b** mass spectrum of DMDS derivative of (*Z*)-9-heptacosene

Triacontanyl Acetate This compound is characteristic of *M. scutellaris* wax and it is not found in *M. rufiventris*. Electron impact mass spectrometry (70 eV), *m/z* (%): 480 ($M^{+\cdot}$, 3%), 420 (8%), 392 (7%), 264 (4%), 125 (35%), 111 (58%), 97 (95%), 83 (97%), and 57 (100%). ^{1}H NMR (300.07 MHz, $CDCl_3$): δ 0.90–1.82 ppm (*m*, 59 H), 2.08 (*s*, 3H. characteristic of methyl belonging to an acetyl group), and 4.06 (*t*, 2H, *J*=8 Hz, hydrogens of a methylene linked to oxygen). ^{13}C NMR (DEPT 135° and 90°, 75.5 MHz, $CDCl_3$): δ 14.2 ppm (CH_3), 21.5 (CH_3), 22.0–34.0 (CH_2), and 65.0 (CH_2 linked to an oxygen).

Invaded Colony The wax composition of *M. rufiventris* invader workers [*n*-alkanes (41.18%), *n*-9-alkenes (38.41%), and alkadienes (7.99%)] barely differed from that in the pure colony, with the total alkane/alkene/alkadiene ratio being maintained (Fig. 2a, b, Table 1). However, the relative abundances of some specific hydrocarbons increased after invasion.

In the wax of *M. scutellaris*, the relative abundances of *n*-alkanes and *n*-9-alkenes were substantially affected by the *M. rufiventris* invasion (Table 1). Compared to the pure colony of *M. scutellaris*, there was a decrease in the relative abundances of *n*-alkanes (3.24%) and *n*-9-alkenes (4.20%), and an increase in triacontanyl acetate (82.27%).

The position of the double bond in *n*-alkenes from wax and batumen samples was determined at C-9, by derivatization with DMDS. Their characteristic fragment ion was the *m/z* 173 $[CH_3(CH_2)_7CH{=}SCH_3]^+$ (Fig. 3a, b, Tables 1 and 3) (Buser et al. 1983; Vincenti et al. 1987). The *n*-9-alkenes (C_{25}–C_{31}) present in both batumens and waxes were identified as *Z* isomers by comparing the values of the ECL (ECL=RI/100) with those published in the literature (Howard and Baker 2003).

Nest Batumens Batumens of both pure colonies and the invaded colony had complex mixtures that were separated by silica column chromatography into fractions (A–E) of increasing polarities (from hexane to ethyl acetate) (Table 2). The components of each fraction, as identified by GC-MS, are shown in Table 3.

Fractions A of the batumen samples from pure colonies have similar size and composition (Tables 2 and 3). The main constituents are *n*-alkanes and *n*-alkenes ranging

from C_{14} to C_{30}. However, fraction A from the invaded colony is 30% larger than that of fraction A from pure colonies (Table 2), and is composed of 13.4% nonoxygenated sesquiterpenes, which are almost absent in fraction A from pure colonies (Table 3).

Fractions B, C, D, and E from pure colonies have similar weights (Table 2), whereas in the invaded colony they weigh 2–30 times more.

The *n*-alkanes ranging from C_{14} to C_{29} in fraction B are present only in this fraction from pure colonies (Table 3). In contrast, triterpenes and triacontanyl acetate are present in fraction B from the invaded colony and the pure colony of *M. scutellaris*. The major triterpene was identified as cycloart-24-en-3β-ol; the identity was established by mass spectral comparison and coinjection in the GC-MS with a standard previously isolated from *Maxillaria friedrichsthalii* (Orchidaceae) floral waxes (Flach 2005).

In the pure colonies of *M. scutellaris* and *M. rufiventris*, fraction C is composed of *n*-alkanes ranging from C_{13} to C_{29}, whereas the fraction C from the invaded colony has no *n*-alkane, and its major constituent (80%) is an unknown triterpene that is not present in the pure colonies (Table 3).

Fraction D from the invaded colony contains resorcinol derivatives, but these are not found in pure colonies.

Fraction E shows many unidentified compounds in the pure colony of *M. scutellaris* (Table 3), but in the pure colony of *M. rufiventris* it has long chain fatty acids, and in the invaded colony mainly phenol derivatives.

Discussion

Our data show that the abdominal waxes of both bee species are chemically similar, especially regarding *n*-alkanes and *n*-alkenes. These were possibly responsible for the "pacific" invasion of *M. scutellaris* nest by *M. rufiventris* workers where the invaders lured the *M. scutellaris* nest guard bees. Our chemical findings corroborate the Roubik's hypothesis that scent similarities between the species could be responsible for this invasion (Roubik 1989). However, some differences in wax composition were also evident, as demonstrated by triacontanyl acetate, which is characteristic of *M. scutellaris* wax and was not present in *M. rufiventris* wax (Table 1). Alkadienes are characteristic of *M. rufiventris* wax and were not detected in the wax of *M. scutellaris*.

As chemistry plays an important role in bee behavior, one could predict that nest sharing between *M. rufiventris* and *M. scutellaris* might produce a chemical response. Indeed, the wax composition of *M. scutellaris* workers from the invaded colony showed an increase in the relative abundances of triacontanyl acetate and a decrease of *n*-alkanes and *n*-9-alkenes when sharing the colony with *M. rufiventris* invader workers (Table 1). On the other hand, chemical changes in *M. rufiventris* wax composition were small. Workers from both pure and invaded colonies displayed almost the same amounts of *n*-alkanes, *n*-9-alkenes, and alkadienes. A chemical response of *M. rufiventris* workers to nest invasion, however, is demonstrated by subtle changes in the relative abundance of each alkane, alkene, and alkadiene. The chemical changes in both bee species in the invaded colony are likely connected to the fact that each species needs to distinguish each other to maintain identity. Such chemical alterations confirm that the invasion is not as "friendly" as first assumed by us, and that *M. scutellaris*, the invaded species, responds more dramatically to the invasion.

In this context, Meliponinae nest construction and habits must be understood. Stingless bees prefer cavities in trees for nest construction; nests are found often in small cavities

(Nogueira-Neto 1997). The bees do not enlarge the cavities, they just use them as they are. In the present case, the colonies were established in manmade wooden boxes, and *M. rufiventris* and *M. scutellaris* constructed their nests with batumen, the mixture of wax, mud, and floral materials, that is used to line the nest, leaving a single hole leading into the nest cavity (Roubik 1989; Nogueira-Neto 1997). The batumens of pure colonies of *M. rufiventris* and *M. scutellaris* are composed of similar amounts of secondary metabolites, as demonstrated by the weight similarity of the batumen fractions A–D (Table 2). However, the change observed when *M. scutellaris* is invaded by *M. rufiventris* is significant, as assessed by the 2–30 fold increase in the weights of fractions B–E in the invaded colony. These changes reflect a higher foraging activity in the invaded colony in comparison with the pure ones.

In addition, the chemical composition of the batumen fractions D–E in the invaded colony has substantial amounts of phenolic derivatives not found in batumens from pure colonies (Table 3). Phenol and resorcinol derivatives could be derived from Anacardiaceae plant species (Aguilar-Ortigoza et al. 2003), widely spread around the Aretuzina farm where the nests are located. *Melipona scutellaris* workers probably collect substances from these plants and deposit them in the nest. This may be evidence of a chemical war in which *M. scutellaris* is stimulated to work more intensively in the nest than *M. rufiventris*. First, there is an increased foraging activity in the invaded colony as revealed by the substantial increase in weight of fraction B of the batumen relative to pure colonies (this quantitative change was not accompanied by a qualitative change in the triterpenoid constituents, which remained approximately the same) (Table 3). Second, fraction E indicates that *M. rufiventris* contributes minimally to the batumen of the invaded nest; in the pure colony of *M. rufiventris*, this fraction contains some long-chain fatty acids that are not found in batumens of either pure or invaded colonies of *M. scutellaris*.

In summary, we found that *M. scutellaris* and *M. rufiventris* are chemically distinguished by their wax composition. *Melipona scutellaris* workers are characterized by having triacontanyl acetate as a specific marker in their abdominal waxes, which they deposit in batumen, whereas *M. rufiventris* workers produce a wax characterized by alkadienes. When *M. rufiventris* workers invade a colony of *M. scutellaris*, physiological changes occur in both species, but the most prominent chemical changes are found in the abdominal waxes of *M. scutellaris*. The data support the idea that the invasion is not pacific, but rather that *M. scutellaris* workers are induced to deposit different substances in the batumen, possibly thus deterring the *M. rufiventris* invader workers.

Acknowledgments We thank Fundação de Amparo a Pesquisa do Estado de São Paulo for grants, which made the present contribution possible (Adriana Flach, Proc. no. 02/07029-0, and Adriana Pianaro, Proc. no. 03/09358-3). We also acknowledge Prof. Carol H. Collins (IQ/UNICAMP) for critically revising the manuscript.

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J Chem Ecol (2007) 33:985–995
DOI 10.1007/s10886-007-9272-7

A 4-Alkyl-substituted Analogue Of Guaiacol Shows Greater Repellency To Savannah Tsetse (*Glossina* spp.)

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Received: 16 August 2006 / Revised: 27 November 2006 / Accepted: 10 December 2006 /
Published online: 3 April 2007
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Abstract The responses of *Glossina morsitans morsitans* Westwood to guaiacol (2-methoxyphenol), a mild repellent constituent of bovid odors, and seven analogues comprising 2-methoxyfuran, 2,4-dimethylphenol, 2-methoxy-4-methylphenol (4-methylguaiacol), 4-ethyl-2-methoxyphenol (4-ethylguaiacol), 4-allyl-2-methoxyphenol (4-allylguaiacol; eugenol), 3,4-methylenedioxytoluene, and 3,4-dimethoxystyrene were compared in a two-choice wind tunnel. The 4-methyl-substituted derivative (2-methoxy-4-methylphenol) was found to elicit stronger repellent responses from the flies compared with guaiacol. None of the other analogues showed significant repellent effects on flies. 4-Methylguaiacol, guaiacol, and eugenol (which was included because of previous reports of its repellency against a number of arthropods) were further evaluated in the field with wild populations of predominantly *Glossina pallidipes* Austen. The presence of guaiacol or eugenol near odor-baited traps caused some nonsignificant reduction in the number of tsetse catches at relatively high release rates (~50 mg/hr). In contrast, the 4-methyl derivative at three different release rates (2.2, 4.5, and 9.0 mg/hr) reduced trap catches of baited traps in a dose-response manner. At 10 mg/hr release rate, it reduced the catches of baited and unbaited traps by ~80 and ~70%, respectively. In addition, the compound not only reduced the number of tsetse attracted to natural ox odor (~ 80%), but also had an effect on their feeding responses, reducing the proportion that fed on an ox by more than 80%. Our study shows that the presence of a methyl substituent at the 4-position of guaiacol enhances the repellency of the molecule to savannah tsetse and suggests that 4-methylguaiacol may represent a promising additional tool in the arsenal of techniques in trypanosomiasis control.

Keywords Tsetse fly · *Glossina pallidipes* · Repellents · Behavior · Trypanosomiasis · Guaiacol · 4-Methylguaiacol

Introduction

Tsetse-transmitted trypanosomiasis continues to cause morbidity and death in human populations and livestock throughout sub-Saharan Africa. Current intervention options

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include parasite control through the use of trypanocidal drugs (Jordan 1986; Holmes, 1997); the promotion of trypanotolerant livestock (FAO 1987a, b; d'Lteren et al. 1999); vector control through the use of visual and odorant baits such as traps (Hassanali et al. 1986; Brightwell et al. 1987, 1991), insecticide-treated targets (Vale et al.1988a), and insecticide-treated animals (Bauer et al. 1995; Hargrove et al. 2000); and sterile insect technique (SIT) (Vreysen et al. 2000).

The potential of repellents to protect cattle and thereby reduce cases of trypanosomiasis has been recognized since the 1970s. Earlier work by Vale (1974a) showed that constituents in human odor reduced the number of tsetse attracted to a host and the number that subsequently fed. The effect was greater on females than on males, and *Glossina pallidipes* was more strongly affected than *Glossina morsitans.* During the process of screening for tsetse attractants, a number of compounds were discovered that had varying levels of repellency against the two savannah tsetse species. These included two synthetic compounds, acetophenone and caproic acid, and two naturally occurring constituents of cattle and human odor, respectively, guaiacol (2-methoxyphenol) and lactic acid (Vale 1977a, 1980; Vale et al. 1988b). Commercially available insect repellents such as N,N-diethyl-meta-toluamide (DEET) and naphthalene were ineffective (Vale 1977a; Torr et al. 1996). Torr et al. (1996) evaluated the effects of controlled release of acetophenone, 2-methoxyphenol, and a series of fatty acids on attractants-baited trap catches of *G. pallidipes* in Zimbabwe. 2-Methoxyphenol reduced the catch by 85%, whereas acetophenone, pentanoic acid, or hexanoic acid halved the respective catches. Further studies on targets baited with a synthetic attractant blend, a source of natural ox odor, and an ox placed at the center of rings of electrified nets showed that whereas 2-methoxyphenol reduced the numbers of tsetse attracted to the attractant sources by ~50% and ~40%, respectively, only pentanoic acid had a small but significant effect on the feeding responses of the flies that arrived closer to the ox. These findings led these authors to conclude that the repellents could not provide any useful degree of protection against trypanosomiasis.

We have explored two approaches to discovering more potent tsetse repellents: (1) by detailed analyses of odors and assays of different blends of candidate semiochemicals of wild animals that are refractory to tsetse (Gikonyo et al. 2000, 2002, 2003); and (2) through structure-activity studies of known natural repellents in the laboratory followed by field evaluations of promising analogues. Herein, we report our discovery of a synthetic analogue of guaiacol (2-methoxyphenol), which has demonstrated greater repellency than the parent compound.

Methods and Materials

Laboratory Bioassays

Wind Tunnel Bioassays Tests were conducted in a two-choice cylindrical plexi-glass tunnel (180 cm long, 24 cm diam) described in detail by Gikonyo et al. (2003). The middle of the tunnel was connected to a PVC pipe in the terminal end of which an air-extracting fan was mounted. The duct in the middle divided the tunnel into two equal arms with a 20-cm wide middle zone where air from both arms mixed. When the fan was switched on, air flowed into the tunnel from both arms, thereby making the middle of the tunnel downwind. The upwind ends of the tunnel were closed with PVC gauze and the down end with a metallic mesh cover. The upwind ends were connected to air filters containing activated charcoal (4-14 mesh, Sigma Chemical Co.) The wind tunnel had three windows, one on each arm (15×10 cm) for

introducing the test odors and one in the middle (4.7 cm diameter) for introducing an insect release cage. Light from fluorescent tubes and bulbs controlled with a dimmer switch was diffused through frosted glass sheeting placed 35 cm above the tunnel, giving about 1,000 lx incident light. A white sheet of paper marked with black stripes about 2 cm apart was placed beneath the tunnel floor to provide contact to a fly during anemotaxis. The wind speed inside the tunnel was maintained at 10 cm/sec. The bioassay room was maintained at 25±2°C and 70± 5% relative humidity (RH).

Test Compounds Guaiacol (2-methoxyphenol) and seven analogues comprising 2-methoxyfuran, 2,4-dimethylphenol, 2-methoxy-4-methylphenol (4-methylguaiacol), 4-ethyl-2-methoxyphenol (4-ethylguaiacol), 4-allyl-2-methoxylphenol (4-allyllguaiacol; eugenol), 3,4-methylenedioxytoluene, and 3,4-dimethoxystyrene (Fig. 1) (each 98–99% pure, obtained from Sigma-Aldrich, Taufkirchen, Germany) were each tested against a control (blank). Eugenol was specifically included in the tests because of previous reports of its repellency against some arthropods (Chogo and Crank 1981; Hassanali et al. 1990; Mwangi

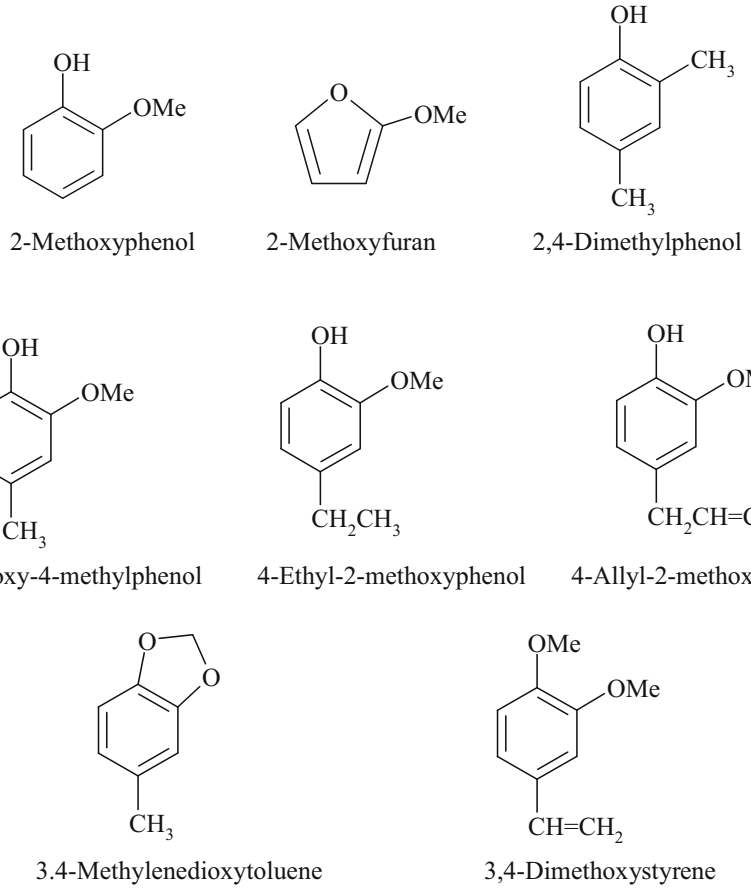


Fig. 1 Structures of 2-methoxyphenol (guaiacol) and analogues screened in the wind tunnel for repellency against *G. m. morsitans*

et al. 1995). A blank test comprising of two blank odor dispensers placed at both upwind ends was also undertaken.

Odor Dispensers The odor dispenser consisted of a black cloth that was secured with Soxhlet-cleaned (dichloromethane) rubber bands placed on one end of an open-ended plexiglass tube (5 cm long and 4.5 cm diam). The dispensers were each placed via the window on the upper side of either arm of the tunnel onto a metallic rack positioned 15 cm from the upwind end such that they were at midheight with the cloth facing downwind. The appropriate dose of the odor was pipetted onto the center of the cloth on one of the dispensers, and air was allowed to flow at 10 cm/sec. A similar dispenser placed in the other arm of the tunnel served as a control. All windows of the tunnel were closed and air allowed to flow for 5 min before the first fly was introduced.

Testing Procedure The procedure detailed in Gikonyo et al. (2003) was followed. One hour before the bioassay, individual flies (3-d-old teneral male tsetse flies) were placed in cylindrical plastic cages (8.0 cm long and 4.5 cm diam) closed on one side with a paper stopper inserted into a semicircular slit. At the start of the bioassay, a release cage containing a quiescent male fly was introduced cautiously through the release hole in the middle of the tunnel (downwind end, middle zone), and the paper plug of the release cage was removed gently so that only air from the charcoal filters carrying odor from the dispensers passed over the release cage. The behavior of each fly was observed and recorded for 3 min, after which the fly was aspirated out with a vacuum pump. The following observations were made: (1) number of flies departing from the midsection; (2) initial direction of flights upwind and any subsequent changes; and (3) return downwind flights and final landing/resting position (middle, control, or treated arms of the tunnel).

In each replicate cycle, involving one dose of a given test odor in 1 day, the above observations on about 10 individual flies were recorded after which the tunnel, the metallic racks, and the release cages were cleaned with water and then with 70% ethanol. With the humidifier switched off, the bioassay room temperature was raised to above 30°C, and air was exhausted from the room for about 6 hr by a powerful extraction fan fixed on the room wall. At the same time, the tunnel was exhausted at maximum wind speed overnight. Blank tests were conducted to confirm no residual effects of previous test materials. Once a week, the tunnel walls were also flushed with hot air from a heat gun. Between three and 10 replicates (corresponding to 30–100 flies observed) were carried out for each treatment. To avoid possible bias, the arms of the tunnel were used alternately for control or test odor in successive replicates. The percent responses were rank-transformed before being subjected to analysis of variance (ANOVA) by using PROC GLM (SAS institute, 2002) with significance level set at 5%.

Insects Newly emerged male *G. morsitans morsitans*, available from the ICIPE colony maintained in a rearing room at 25±2°C, 65±5% RH on a 12:12 (L/D) photocycle, were used as a representative of savannah morsitans species to initially screen the relative repellency of the test compounds in the laboratory.

Field Experiments

Field Site All experiments were carried out at ICIPE's Nguruman field station, southwest of Kenya where *G. pallidipes* Austen are predominantly present.

Traps All catches were made by NG2G traps, baited with acetone at ~500 mg/hr and cow urine aged 1–4 wk at overall (water plus volatiles) release rate of ~1,000 mg/hr, which provide relatively uniform catches (Brightwell et al. 1991). Traps were placed 100 m apart along the forest edge. The odors were placed on the ground 30 cm downwind of the trap.

Effects of Various Candidate Repellents on Trap Catches The effect of 2-methoxyphenol (guaiacol), 4-allyl-2-methoxyphenol (eugenol) and 2-methoxy-4-methylphenol (4-methyl-guaiacol) on trap catches were compared in a series of Latin square design experiments comprising of the following treatments:

(a) Baited trap, baited trap with 1.0 or 10.5 mg/hr eugenol, and baited trap with 1.0 or 9.0 mg/hr guaiacol, respectively. In the second experiment, higher doses (25.0 or 50.0 mg/hr) of the two compounds were evaluated. The repellents were dispensed undiluted from glass vials (1.7 cm wide and 4.3 cm deep) with lids containing 1- or 2-mm-diam holes to give the desired release rates.
(b) Unbaited trap, baited trap, baited trap with 2-methoxy-4-methylphenol at a dose of 10.0 mg/hr. The repellent was dispensed from glass vials, as above.
(c) Baited traps with 2.2, 4.5, or 9.0 mg/hr, 2-methoxy-4-methylphenol. In this set of experiments, the repellent was dispensed from sealed polythene sachets (25–50 cm^2 surface area and 50 μm thickness). The surface area of the sachets was varied to obtain the different release rates.

In all the above experiments, the repellents were incorporated into a series of replicated Latin squares consisting of days × sites × treatments. The catches (n) were normalized by using a $\log_{10}$ (n + 1) transformation and subjected to analysis of variance (ANOVA with PROC GLM, SAS Institute, 2002). The detransformed means are reported accompanied by their transformed means and standard errors.

4-Methylguaiacol Effects on Glossina pallidipes Responses to Natural Attractants This set of experiments was conducted to determine the number of tsetse attracted to a source of ox odor with or without the synthetic repellent. The odor was generated by placing an ox (~250 kg) in a pit (5 m long, 2 m wide, and 2 m deep with iron sheet roofing and entrance door) and exhausting the air from the pit at 2,000 l/min via a ventilation shaft fitted with a 12-V co-axial fan at the end (Vale 1974b). Two such pits were constructed. To estimate the number of tsetse attracted to the odor, an electric net (1.0×1.0 m) was placed 1 m downwind of the odor source (Vale 1974b). Sheets of corrugated fiberglass coated with a thin film of polybutene were placed underneath the screens to collect the electrocuted flies. As tsetse orientate imprecisely to an odor source in the absence of a visual stimulus (Vale 1974b), a visual model (metal drum 50 cm long and 37 cm diam covered with black cloth) was placed between the screen and odor source. A balanced incomplete Latin square design was used for these experiments with the following treatments: (1) ox odor, (2) ox odor + model, (3) ox odor + repellent, (4) ox odor + model + repellent. The repellent (10 mg/hr) was released from a polythene sachet placed near the odor outlet. The experiments were conducted for 3 hr in the morning (0800–1100 hours) and 3 hr in the afternoon (1400–1700 hours).

Effect of 4-Methylguaiacol on Feeding Efficiency of Glossina pallidipes The feeding efficiency of *G. pallidipes* on an ox with or without the repellent was determined by using the method developed by Vale (1977b). In this experiment, an ox was tethered in the middle of an incomplete ring of five electric screens (1.0×1.0 m). The screens covered 20% of the circumference of the circle. These rings were constructed at two different locations. The

Table 1 Behavioral responses of male teneral *Glossina mositans morsitans* to guaiacol and analogues in a wind tunnel

Test Odor	Dose (μl)	*N*	Percent files departing from midsection	Final resting position			
				Control	Center	Treated	*P*
2-methoxyphenol (guaiacol)	100	100	45.8	60.90±5.99a	3.33±3.33[c]	35.80±8.52b	0.01
	200	119	57.1	40.63±11.78	27.50±8.28	31.90±11.68	Ns
	400	88	58.1	46.25±8.69	20.72±5.23	33.05±5.80	Ns
2-methoxyfuran	100	40	62.5	46.65±9.71	21.48±6.25	31.87±5.24	Ns
	200	30	60	36.47±5.87	40.37±14.91	23.13±12.85	Ns
	400	30	76.67	20.83±6.35	40.27±5.01	38.87±5.57	Ns
2,4-dimethylphenol	100	30	53.33	49.03±4.96	18.10±11.7	32.87±8.92	Ns
	200	30	50	38.43±14.35	20.93±12.36	40.63±9.78	Ns
	400	30	66.67	39.53±6.18	35.93±5.53	24.53±2.49	Ns
2-methoxy-4-methylphenol	25	80	65	30.13±5.71[a]	63.53±5.70[b]	6.35±3.22[c]	0.001
	50	71	64	30.83±7.68[ab]	56.78±11.29[a]	12.38±4.59[b]	0.05
	100	80	65	56.24±11.93[a]	25.23±7.03[b]	18.54±5.21[b]	0.05
	200	80	76.5	53.56±6.03[a]	21.83±6.23[b]	24.61±2.69[b]	0.001
	400	70	75	63.53±5.70[a]	15.99±4.83[b]	20.59±3.45[b]	<0.001
4-ethyl-1,2-dimethylphenol	100	35	62.9	26.20±14.48	28.7±14.85	45.2±4.77	Ns
	200	24	83.3	75.00±1.00	12.5±5.5	12.5±12.5	Ns
	400	30	70.3	27.70±1.82	41.7±7.20	30.6±5.60	Ns
4-allyl-2-methoxyphenol	100	30	50	38.90±20.04	19.43±10.01	41.67±30.05	Ns
	200	30	56.7	6.67±6.67	49.00±24.79	44.43±29.39	Ns
	400	30	62.5	24.47±2.68	43.43±7.05	32.10±8.60	Ns
3,4-methylenedioxytoulene	100	30	76.67	40.73±15.44	12.17±6.49	47.10±9.26	Ns
	200	30	73.33	48.53±4.56[a]	14.53±2.76[b]	36.93±1.95[a]	0.05
	400	30	36.67	24.50±15.28[a]	11.50±7.58[ab]	64.00±15.28[c]	0.01
3,4-dimethoxystyrene	100	35	56.7	52.8±13.90	11.1±11.1	36.1±21.69	Ns
	200	34	46.7	53.9±17.12	32.8±4.34	13.3±13.33	Ns
	400	30	65	29.0±5.51	19.4±1.00	51.7±10.27	Ns

experiment was a randomized complete block design and treatments were: (1) ox alone and (2) ox + repellent, which were randomly assigned to the two sites. At the site that received the repellent treatment, sachets containing the repellent (10 mg/hr) were placed on the post to which the animal was tied. The animals were kept stationary, whereas the treatments were rotated after each cycle of the experiment. Because in a preliminary experiment a relatively higher number of flies were caught in the afternoon, all the experiments were run for 3 hr from 1500–1800 hours. At the end of the experiment, the numbers of flies caught inside and outside the ring were recorded. Flies were also classified as fed or unfed based on the presence of fresh blood visible through the abdominal wall. The number of flies approaching the target (animals) with/without a repellent were estimated and statistically analyzed. Following Vale (1977b), feeding efficiency was estimated as a percentage of the fed tsetse relative to the total catch (fed + unfed) inside the ring of nets.

Results

Responses in the Wind Tunnel Equal number of flies flew upwind to the control (44%) or treated (47%) arms of the wind tunnel (N=145) when no odor was dispensed (blanks). Table 1 shows the responses of *G. m. morsitans* to the eight methoxyphenol analogues tested in the two-choice wind tunnel. Only the final resting position was statistically affected by the presence of a candidate repellent (Table 1) as previously shown with waterbuck repellents, a refractory host of savanna tsetse (Gikonyo et al. 2003). Only one 4-substituted methoxyphenol derivative, 2-methoxy-4-methylphenol, elicited consistent and significant repellent responses. The final resting position was dependent on the dose of this phenol: at lower doses (25 and 50 μl), more of the flies flying downwind settled in the midsection of the tunnel; however, at higher doses (100–400 μl) the flies tended to fly further away from the repellent-treated side and landed on the control arm of the wind tunnel (no odor present). Guaiacol (2-methoxyphenol) itself elicited milder responses, and

Table 2 Mean catches (detransformed and transformed ± SE) of *Glossina pallidipes* from NG2G-baited (cow urine + acetone) traps with various doses of eugenol or guaiacol (N=10)

Treatment	Trap catch	
	Detransformed	Transformed (±SE)
Expt 1		
Baited trap (BT)	524.807	2.720±0.138
BT + 1.0±0.18 mg/hr eugenol	644.169	2.809±0.137
BT + 10.5±0.03 mg/hr eugenol	516.416	2.173±0.140
BT + 0.9±0.27 mg/hr guaiacol	347.536	2.541±0.125
BT + 9.18±0.19 mg/hr guaiacol	218.273	2.339±0.104
Expt 2		
Baited trap (BT)	368.978	2.567±0.095
BT + 25.7±0.16 mg/hr eugenol	246.604	2.392±0.119
BT + 50.2±0.03 mg/hr eugenol	157.036	2.196±0.091
BT + 24.36±0.28 mg/hr guaiacol	285.759	2.456±0.137
BT + 49.81±0.17 mg/hr guaiacol	220.800	2.344±0.102

Table 3 The detransformed mean catch (transformed ± SE in brackets) of *Glossina pallidipes* from unbaited or baited (cow urine + acetone) NG2G traps plus 4-methylguaiacol

Treatment	Mean catch	Index	Percent reduction
Unbaited trap	214.2 (2.333±0.070)*	0.29×	71
Baited trap	740.7 (2.870±0.048)	1.0×	
Baited trap + repellent	138.8 (2.146±0.050)*	0.19×	81

The catch index is the detransformed mean catch of tsetse expressed as a proportion of that from a baited trap (N=24)

The asterisk indicates that means are significantly different from baited trap catch (P<0.05, F test)

in one dose (100 μl), the number of flies that flew downwind and settled on the control arm was statistically significant. Contrary to our expectation, eugenol did not elicit any significant repellent responses from *G. m. morsitans*.

Effect of Candidate Repellents on Baited Trap Catches In field experiments, neither 2-methoxyphenol (guaiacol) nor 4-allyl-2-methoxyphenol (eugenol) reduced the trap catches of *G. pallidipes* ($F_{8,41}$=0.13 and $F_{8,41}$=0.43, respectively, Table 2).

The inclusion of 4-methylguaiacol with unbaited and baited (acetone + cow urine) traps reduced trap catches (>70 and 80%, respectively) ($F_{4,67}$ =31.38; P<0.001, Table 3). There was no significant difference in the responses of males and females, and hence the data for the sexes were pooled.

Effect of 4-Methylguaiacol on Attraction to Natural Attractants In both experiments, *G. pallidipes* catches to ox odor (ox placed in a ventilated pit) with 4-methylguaiacol were reduced by ~80% ($F_{3,20}$=18.54, P<0.001 and $F_{3,20}$= 100.77, P<0.001, respectively, Table 4). In the presence of the visual stimulus (metal drum covered with black cloth placed between the screen and odor source), the repellent caused reductions of ~70 and ~80% in the two experiments, respectively.

Table 4 The detransformed mean catch (transformed ± SE in brackets) of *Glossina pallidipes* from a target at a source of natural ox odor with and without 4-methylguaiacol

Treatment	Mean catch	Index	Percent reduction
Expt 1			
Ox odor	25.6 (1.424±0.093)	1.0×	
Ox odor + model	31.8 (1.516±0.092)ns	1.24×	
Ox odor + repellent	5.3 (0.799±0.082)*	0.20×	80
Ox odor + model + repellent	7.9 (0.949±0.085)*	0.31×	69
Expt 2			
Ox odor	358.1 (2.555±0.354)	1.0×	
Ox odor + model	379.3 (2.580±0.036)ns	1.06×	
Ox odor + repellent	66.8 (1.831±0.031)*	0.19×	81
Ox odor + model + repellent	64.7 (1.818±0.062)*	0.1×	82

The catch index is the detransformed mean catch of tsetse expressed as a proportion of that from an ox odor source (N=6)

The asterisk indicates that means are significantly different from ox odor (P<0.05, F test)

Table 5 The detransformed mean catch (transformed ± SE in brackets) of *Glossina pallidipes* from NG2G-baited (cow urine and acetone) traps with various doses of 4-methylguaiacol

Treatment (baited trap + repellent)	Mean catch	Index	Percent reduction
A Trap alone (control)	117.8 (2.075±0.084)[a]	1×	
B Trap + 2.2 mg/hr	41.6 (1.629±0.071)[b]	0.35×	65
C Trap + 4.5 mg/hr	38.6 (1.598±0.068)[b]	0.33×	67
D Trap + 9.0 mg/hr	29.8 (1.489±0.071)[b]	0.25×	75

The catch index is the detransformed mean catch of tsetse expressed as a proportion of that from a baited trap (control) (N=16). Means followed by different letters are significantly different (P<0.05, F test)

Effect of 4-Methylguaiacol on Trap Catches All release rates of 4-methylguaiacol significantly reduced trap catches, with 9.0 mg/hr showing maximum reduction ($F_{3,60}$=12.61, P<0.001, Table 5).

Effect of 4-Methylguaiacol on Feeding Efficiency of Glossina pallidipes on Host The studies on feeding levels of *G. pallidipes* in the field (Table 6) showed that in addition to reducing the proportion of the tsetse attracted to an ox by >80% (Table 4), the candidate repellent also reduced host feeding from 27 to 4.8%, a >80% reduction.

Discussion

In a previous study undertaken in Zimbabwe (Torr et al. 1996), guaiacol at 5–10 mg/hr reduced the numbers of *G. pallidipes* attracted to baited and unbaited Epsilon (Hargrove and Langley 1990) traps (~85 and ~60%, respectively), baited targets (~50%), and a source of natural ox odor (~40%). In the present study, the compound elicited relatively mild repellent responses from *G. m. morsitans* in a two-choice wind tunnel, but no significant reductions in baited trap catches of *G. pallidipes* even at relatively high doses. The difference in the two studies may be due to different efficiency of the two trap designs used and/or some differentiation in the sensory responses of the two *G. pallidipes* populations. Detailed comparison of the electrophysiological and behavioral responses of the two *G. pallidipes* populations and their close-range behaviors to the two traps may shed some light on the question.

Despite the relatively high repellency demonstrated in different experiments in Zimbabwe, guaiacol had no significant effect on the numbers of flies that fed on an ox, which makes it an ineffective tool for protecting cattle against trypanosomosis (Torr et al.

Table 6 Mean catches and percent feeding efficiency of *Glossina pallidipes* on cattle with and without 4-methylguaiacol

Treatment	Total catch (inside the screen)	Total catch (inside and outside the screen)	Total fed (inside the screen)	Feeding efficiency (%)
Ox alone	445±8.5[a]	818±23.5[a]	120±10.3[a]	27
Ox + repellent	83±4.5[b]	154±9.6[b]	4±0.2[b]	4.8

Synthetic repellent reduces the feeding efficiency on treated cattle by 82%. Feeding efficiency is the catch of fed flies from inside the ring of nets expressed as proportion of the total catch (fed + unfed). Number of replicates = 6. Means followed by different letters are significantly different at (P<0.05, F test)

1996). In the present study, the presence of a methyl substituent at the 4-position of guaiacol significantly enhanced the repellency of the molecule to savannah tsetse *G. m. morsitans* in the wind tunnel, and reduced numbers of *G. pallidipes* attracted to baited and unbaited traps and to ox odor. In addition, the compound reduced the number of flies that landed on the ox and those that fed on the host (>80%).

These results suggest that 4-methylguaiacol represents a promising tool for protecting individual cattle against trypanosomiasis (Saini and Hassanali 2004). We are currently exploring two strategies for integrating the repellent with existing tools and tactics. First, we are studying its efficacy in controlled release devices carried by individual cattle owned by nomadic communities who need a mobile technology to protect their cattle and reduce their dependence on trypanocidal drugs. Such devices may also provide an effective means of reducing challenge to trypanotolerant cattle, which succumb to the disease at high infection rates (Jordan 1986). Second, use of the repellent as a "push" component with bait technologies (traps, targets, or live baits with insecticides as "pull") in "push–pull" tactics in areas where cattle are the dominant source of blood meal may provide a more effective way of suppressing tsetse among settled pastoralist and agro-pastoralist communities.

The enhanced repellency of 4-methylguaiacol (and lack of activity of the other analogues tested) against savannah tsetse discovered in the present study raises some interesting questions about the sensory mechanism that underlies its action. This phenol reduced the catch of *G. pallidipes* in unbaited traps that were only visually attractive and thus, according to Dethier et al. (1960) definition, qualifies as a true repellent. This suggests that these phenols probably interact with a specific group of receptors different from those of the two tsetse kairomonal phenols, 4-methylphenol and 3-*n*-propylphenol (Owaga et al. 1988; Saini and Hassanali 1992). They not only inhibit or counter the response of sensory signals from the receptors of these attractants, but also those associated with close-range visual attraction (Davis 1985). However, the close structural relationship between 4-cresol and 4-methylguaiacol and lack or mild activity of the respective parent phenols and those of ethyl analogues (Owaga et al. 1988) suggests a close similarity of the binding sites of their respective receptors. Our current studies on the odor binding proteins of savannah tsetse are expected to provide some insights into the relationship between the two sets of receptors and their relative specificity.

Acknowledgments This study was carried out with funding from the European Union (EU) and the International Fund for Agricultural Development (IFAD) to whom we are grateful. We thank John Andoke, Peter Musa, Melaku Girma, and Caroline Muya for technical assistance and Anthony Wanjoya for assistance in statistical analyses of data.

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J Chem Ecol (2007) 33:997–1012
DOI 10.1007/s10886-007-9273-6

Induction of Plant Volatiles by Herbivores with Different Feeding Habits and the Effects of Induced Defenses on Host-Plant Selection by Thrips

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Received: 11 September 2006 / Accepted: 23 February 2007 /
Published online: 29 March 2007
© Springer Science + Business Media, LLC 2007

Abstract Induced plant responses to attack by chewing insects have been intensively studied, but little is known about plant responses to nonchewing insects or to attack by multiple herbivores with different feeding habits. We examined volatile emissions by tobacco, *Nicotiana tabacum*, in response to feeding by the piercing–sucking insect western flower thrips (WFT), *Frankliniella occidentalis*, the chewing herbivore *Heliothis virescens*, and both herbivores simultaneously. In addition, we examined the effects of herbivore-induced plant defenses on host-plant selection by WFT. Plants responded to thrips feeding by consistently releasing five compounds. Simultaneous feeding by WFT and *H. virescens* elicited the same 11 compounds emitted in response to caterpillar feeding alone; however, two compounds, α-humulene and caryophyllene oxide, were produced in greater amounts in response to simultaneous herbivory. In choice tests, thrips consistently preferred uninduced plants over all other treatments and preferred plants damaged by caterpillars and those treated with caterpillar saliva over those treated with caterpillar regurgitant. The results are consistent with a previous finding that caterpillar regurgitant induces the release of significantly more volatile nicotine than plants damaged by caterpillars or plants treated with caterpillar saliva. A repellent effect of nicotine on WFT was confirmed by encircling unwounded plants with septa releasing volatile nicotine. Our results provide the first direct evidence that thrips feeding induces volatile responses and indicates that simultaneous herbivory by insects with different feeding habits can alter volatile emissions. In addition, the findings demonstrate that induced plant responses influence host-plant selection by WFT and suggest that the induction of volatile nicotine may play a role in this process.

Keywords Plant–insect interactions · Induced defenses · Plant volatiles · Simultaneous herbivory · Ovipositional preference · Insect behavior · Host-plant selection · *Frankliniella occidentalis* · *Heliothis virescens* · *Nicotiana tabacum* · Nicotine

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Introduction

Herbivory induces plant defense responses including the production of toxic secondary metabolites and the synthesis and release of volatile chemical signals that are attractive to herbivore natural enemies (Turlings et al. 1990, 1995; Karban and Baldwin 1997; De Moraes et al. 1998; Dicke 1999; Pare and Tumlinson 1999). These induced responses can alter host-plant quality and impact the behavior and performance of subsequent herbivores (Karban and Baldwin 1997; Agrawal 1999; De Moraes et al. 2001; Wise and Weinberg 2002; De Moraes and Mescher 2004; Rodriguez-Saona et al. 2005). Interactions between plants and chewing insects (e.g., lepidopteran larvae) are well documented (Karban and Baldwin 1997; Walling 2000). Much less is known about plant responses to insects that employ other feeding modes, such as aphids, whiteflies, and bugs (Du et al. 1998; Turlings et al. 1998; Rodriguez-Saona et al. 2002, 2003; Williams III et al. 2005), although extensive research has been conducted on the spider mite *Tetranychus urticae*, a non-insect arthropod that also feeds in a piercing–sucking manner (Dicke and Sabelis 1988; Dicke et al. 1990a, b). Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), is a piercing–sucking insect and a major worldwide pest of agriculture and horticulture (Lewis 1997; Kirk and Terry 2003). We are unaware of any previous research that has explicitly examined volatile induction in response to WFT feeding, but WFT infestation is known to influence subsequent insect behavior. For example, the predatory bug *Orius laevigatus* prefers cucumber plants infested with WFT (Venzon et al. 1999), whereas the spider mite *T. urticae* avoids them (Pallini et al. 1997). Thrips themselves may exploit cues from infested plants to avoid intraspecific competition (Agrawal and Colfer 2000).

Plant responses to feeding by multiple herbivore species that employ different feeding modes are also poorly understood, although attack by herbivores with diverse feeding strategies is common in nature (Strauss 1991; Vos et al. 2001). Simultaneous feeding by multiple herbivores may influence plant volatile responses by inducing competing plant defense pathways, with possible implications for interactions with the third trophic level. For example, feeding by two species of Lepidoptera, *Plutella xylostella* and *Pieris rapae*, causes cabbage plants to emit different volatile blends from those emitted in response to feeding by either species alone and alters the response of parasitoids (Shiojiri et al. 2001). Additionally, the mixing of different volatile blends emitted from neighboring plants simultaneously infested with different herbivore species has the potential to influence the foraging success of herbivore natural enemies (Dicke et al. 2003).

This paper explores plant volatile responses to feeding by WFT and to simultaneous feeding by WFT and the chewing insect *Heliothis virescens* (Fabricius) on tobacco, *Nicotiana tabacum* L. In addition, we document the impacts of induced plant responses on host-plant selection by WFT. Prior herbivory can influence the behavior of subsequent herbivores by impacting host-plant quality (Karban and Baldwin 1997), by eliciting volatile responses that serve as cues for foraging insects (e.g., De Moraes et al. 2001), and by inducing other biochemical changes in host plants (e.g., Duffey and Stout 1996). We previously found that tobacco plants treated with *H. virescens* regurgitant release significantly more volatile nicotine than plants treated with *H. virescens* saliva or plants damaged by *H. virescens* caterpillars (Delphia et al. 2006). In the current study, we examine the effects of *H. virescens* saliva- and regurgitant-induced plant responses, as well as other treatments, on the distribution and ovipositional preferences of WFT. Our results offer insight into the dynamics of plant–herbivore interactions when plants are simultaneously attacked by herbivores with different feeding habits, and into the role of induced defenses in influencing host-plant choice by an important insect pest.

Methods and Materials

Plants and Insects

Tobacco seeds (*N. tabacum* strain K326) were germinated in a peat-based, general-purpose potting soil (Pro-Mix, Premier Horticulture Inc., Quakertown, PA, USA) and transplanted as 2-wk-old seedlings into pots (16 cm tall × 16.5 cm diam) with fertilizer (Osmocote/ Hummert International, MO, USA) in a growth chamber [16:8 hr light:dark; 25:22°C day: night; 65% relative humidity (RH)]. Five-wk-old plants with three fully expanded leaves were used for all experiments. Eggs of *H. virescens* were obtained from the USDA/ARS Research Laboratory in Tifton, Georgia, and reared on an artificial casein diet in a growth chamber under the same conditions as above. Adult and larval *F. occidentalis* were obtained from infested greenhouse plants that were virus-free (i.e., plants free of symptoms typical for tomato spotted wilt virus: chlorotic rings, chlorotic mosaic patterns, mottled leaves, reduction of growth or deformation).

Induced Plant Volatiles

Simultaneous Herbivory

To examine induced plant responses to herbivory, we collected volatiles from tobacco plants receiving the following treatments: (1) feeding by adult and larval thrips, (2) mechanical wounding, (3) mechanical wounding plus feeding by adult and larval thrips, (4) feeding by three third-instar *H. virescens*, or (5) feeding by three third-instar *H. virescens* plus adult and larval thrips. Undamaged plants were used as controls. Treatments were applied daily, immediately before volatile collection, and volatiles were collected on d 1–4 to ensure induction in response to herbivory. Plants were mechanically wounded by using a razor blade to scrape an approximately 1 cm^2 area on each of the three leaves per plant. The same three leaves received new mechanical damage each day so that by the end of the 4-d experiment, plants had three leaves with four wounds each. Five adult and five first- or second-instar thrips were added to thrips treatments each day so that by the end of the 4-d experiment, 20 adult and 20 larval thrips had been added to each plant. Thrips were added daily to ensure that a minimum number of thrips were feeding each day, as they tended to escape from the volatile collecting chambers. The level of infestation was chosen based on the levels found in greenhouses that employ conservative action thresholds for monitoring thrips. *Heliothis virescens* were allow to feed continuously throughout the 4-d experiment. For treatments where both herbivores were allowed to feed on plants, the feeding location of each herbivore did not appear to be influenced by the presence of the other, and both thrips and caterpillars fed on the same leaves. This experiment was replicated four times.

Increased Levels of Thrips Feeding Damage

To determine if higher levels of thrips damage would alter emission profiles, we collected volatiles from tobacco plants exposed to two additional levels of thrips feeding. We employed (1) a medium-damage treatment where plants received 20 first- or second-instar thrips and 20 adult thrips on the first day of the experiment, followed by 10 additional adult thrips per day for the following 3 d for a total of 70 thrips added to each plant, and (2) a high-damage treatment where plants received 40 first- or second-instar thrips and 40 adult

thrips on the first day of the experiment, followed by 20 additional adult thrips per day for the following 3 d for a total of 140 thrips added to each plant. Thrips were added immediately before volatile collection, and volatiles were collected on each of the 4 d to ensure induction in response to herbivory. This experiment was replicated three times.

Collection and Analysis of Volatiles

Volatiles were collected from tobacco plants by using a closed push/pull system. A two-piece Teflon® base with a hole for the plant stem rested on the pot. A glass dome chamber (15 cm tall×16 cm diam) enclosed plants and rested on the base. Filtered air was pushed through Teflon® tubing into the top of the chamber (3.0 l/min) and was pulled through side ports (0.8 l/min) across beds of adsorbent Super-Q® (25 mg, Alltech Associates, Deerfield, IL, USA). Plant volatiles were collected between 1000 and 2200 hours (light period: 0600 to 2200 hours). Super-Q® traps were rinsed with 150 μl of dichloromethane; 5 μl of *n*-octane (40 ng/μl) and *n*-nonyl-acetate (80 ng/μl) were added as internal standards. Samples were injected, using a splitless injector held at 220°C, in 1-μl aliquots into an Agilent model 6890 gas chromatograph fitted with a flame ionization detector. The column (15 m× 0.25 mm i.d., 0.1 μm film thickness, HP-1) was maintained at 35°C for 0.5 min and then increased by 12°C per min to 180°C. Quantifications of compounds were made relative to the internal standard using ChemStation software (Agilent Technologies, Wilmington, DE, USA). Samples were also analyzed by gas chromatography mass spectrometry by using electron ionization (6890 gas chromatograph interfaced to a Hewlett-Packard 5973N mass selective detector). The column (30 m×0.25 mm i.d., 0.25 μm film thickness, HP-1MS) was maintained at 35°C for 0.5 min and then increased by 12°C per min to 180°C. Identifications of all volatile compounds were confirmed by comparing retention times and mass spectra to commercial standards.

Leaf Measurements

To determine leaf area for plants used for volatile collections, we removed leaves from plants and photocopied them. Photocopies were digitized, and leaf areas and amount of damage were determined using an imaging program (SigmaScan® Pro, SPSS Inc., Chicago, IL, USA).

Collection of *H. virescens* Salivary Glands and Regurgitant

Labial salivary glands were dissected from artificial-diet-fed *H. virescens* larvae that had been fifth-instars for 48 hr and had been chilled on ice for approximately 5 min. Once removed, the salivary glands were placed into microcentrifuge tubes, kept on ice in groups of 10 pairs, and stored at −80°C until needed. Immediately before use, each 10-gland sample was extracted in 50 μl physiologically buffered saline (pH 7.2) by homogenizing the salivary glands with a hand-held pestle in a microcentrifuge tube kept on ice. This allowed salivary-gland extracts to be applied to mechanically wounded leaves with a pipette. Protein concentrations were determined by using the method of Bradford (1976). Total protein concentrations for our samples of homogenized salivary glands were around 700 μg per 40 μl (i.e., the amount applied to plants in experiments). This amount was based on a previously described method (Delphia et al. 2006).

Before collecting regurgitant, recently molted fifth-instar caterpillars were fed for 48 hr on tobacco leaves from approximately 7-wk-old plants. Regurgitant was collected from these larvae by gently squeezing the caterpillars, collecting the resulting oral secretions with a pipette,

and dispensing them into a microcentrifuge tube on ice. Regurgitant was pooled from approximately 50 larvae and then separated into 50 μl aliquots and stored at −80°C until needed.

Thrips Distribution and Ovipositional Preferences

We conducted a series of choice tests to examine thrips behavior in response to induced and uninduced tobacco plants. Adult thrips were allowed to choose between undamaged control plants and plants that received various treatments including mechanical wounding, wounding plus the application of caterpillar saliva or regurgitant, and caterpillar feeding. We also explored the effects of volatile nicotine on thrips behavior by allowing thrips to choose between undamaged plants surrounded by rubber septa releasing nicotine and those surrounded by blank septa. In all choice tests, adult thrips (20–50 depending on the experiment) were released each day for 2 d then counted every day for 3, 4, or 6 d (depending on the experiment) to determine thrips distributional preferences. At the end of all experiments, the leaves of plants were removed, and the numbers of larvae were counted to determine ovipositional preferences. All choice experiments were conducted under greenhouse conditions except for the nicotine choice experiment that was done in a growth chamber.

Effects of Mechanical Wounding

To determine the effects of mechanical wounding on thrips distribution and oviposition preferences, we allowed thrips to choose between wounded plants and unwounded controls. Two mechanically wounded plants and two control plants were positioned 0.3 m from each other at one end of a greenhouse bench (approximately 1.5×3 m), creating a square with wounded plants on one side and control plants on the other. This design was replicated, with treatments reversed, at the other end of the bench. On the first day of the experiment, the plants were wounded by using a razor blade to damage three leaves (as above). This was repeated on d 2–5, wounding the same three leaves each day. On d 1 and 2, wounding was followed by the release of 20–30 adult WFT from a 150-ml beaker in the center of each set of four plants. On d 3–6, we recorded the number of adult thrips on each plant. On d 8, we removed leaves and counted the larvae. Observations and wounding occurred each morning between 0800 and 1000 hours. This experiment was replicated six times.

Effects of Caterpillar Feeding and Oral Secretions

To determine the effects of caterpillar feeding damage and the application of oral secretions associated with herbivore feeding on thrips distribution and ovipositional preferences, thrips were allowed to choose between tobacco plants receiving the following treatments: (1) prior feeding (~18 hr) by two third-instar *H. virescens* followed by continuous feeding by one third-instar *H. virescens* for 5 d, (2) mechanical wounding, (3) mechanical wounding plus the application of 40 μl of *H. virescens*-homogenized salivary glands distributed among three wounds per day, (4) mechanical wounding plus the application of 40 μl of *H. virescens* regurgitant distributed among three wounds per day, or (5) control (i.e., unwounded) plants. We were unable to include a treatment that examined thrips ovipositional preferences in response to plants induced by thrips infestation because of an inability to exclude or distinguish larvae resulting from prior infestation. The amounts of oral secretions applied to plants were based on a previously described method (Delphia et al. 2006).

Five plants were positioned in a circle equidistant from a central point (0.3 m) on each of the two greenhouse benches (approximately 1.5×3 m). The positions of the treatments on each bench were arranged so that no two treatments were adjacent to one another on both benches. On d 1, the plants were wounded by using a razor blade to damage three leaves (as above). Regurgitant and homogenized salivary glands were applied at this time using a pipette to evenly distribute the compounds among the damaged sites. This procedure was repeated on d 2–5. Also, on d 1, we removed one of the two third-instar *H. virescens*, which had been feeding for the previous 18 hr, from the caterpillar-damaged treatment to limit leaf tissue loss. The remaining caterpillar was allowed to feed continually on d 1–5. On d 1 and 2, wounding and the application of oral secretions were followed by the release of 30–40 adult WFT from a 150-ml beaker in the center of each set of five plants. On d 3–8, we recorded the number of adult thrips on each plant. On d 8, we removed leaves and counted the larvae. All observations and manipulations occurred between 0900 and 1100 hours. This experiment was replicated six times.

Effects of Volatile Nicotine

To determine the effects of volatile nicotine on thrips distribution and ovipositional preferences, thrips were allowed to choose between unwounded tobacco plants encircled by rubber septa that had been treated with either nicotine or left blank (i.e., treated with solvent alone). Two plants were positioned about 1 m from each other in a growth chamber (16:8 light:dark; 25:22°C day:night; 65% RH). Three rubber septa, which were previously conditioned with 150 μl dichloromethane, were positioned on metal stands around each of the two plants. With a pipette, 2 μl of nicotine was added to each of the septa in the nicotine treatment. Control septa were left blank. On the first 2 d of the experiment, 45–55 adult WFT were released from a 150-ml beaker centered between the two plants. On d 2, 3, and 4, we recorded the number of adult thrips per plant. On d 7, we removed leaves and counted the larvae. Observations and thrips release occurred each morning between 1030 and 1130 hours. This experiment was replicated three times.

Statistical Analyses

We analyzed the data collected on d 4 from volatile collection experiments to allow sufficient induction in response to herbivory. We quantified all volatile compounds released in measurable amounts. The data from the simultaneous herbivory experiment were $\log_e(x+1)$-transformed to satisfy the assumptions of normality and homogeneity of variance among treatments and were analyzed by analysis of variance (ANOVA), with trial treated as a random effect. The data from the levels of thrips feeding damage experiment were analyzed by ANOVA. All pairwise comparisons were conducted by using Tukey's honestly significant difference (HSD) test. The data from choice tests were square-root-transformed to homogenize variance among treatments. We analyzed both the mechanical wounding and the caterpillar feeding and oral secretions choice tests for adult thrips distribution over four and six sampling days, respectively, by repeated-measures ANOVA, with day treated as a fixed effect and with trial and bench position within trial treated as random effects. We analyzed the volatile nicotine choice test for adult thrips distribution over three sampling days by repeated-measures ANOVA, with day treated as a fixed effect. We used ANOVA to analyze the number of larvae per plant at the end of all three choice tests. All statistical analyses were conducted by using Minitab v. 14.1 (Minitab Inc., State College, PA, USA).

Results

Induced Plant Volatiles

Thrips Feeding Damage

When we compared volatile emissions in response to low, medium, and high levels of thrips feeding damage, we saw an apparent increase in the total amount of volatiles released, although this difference was not statistically significant (ANOVA: $F_{2,\ 7}=2.86$, $P=0.124$; Fig. 1a; Table 1). Plants responded to low levels of thrips feeding by releasing two compounds consistently ((*E*)-β-ocimene and β-caryophyllene; Table 1). Two additional

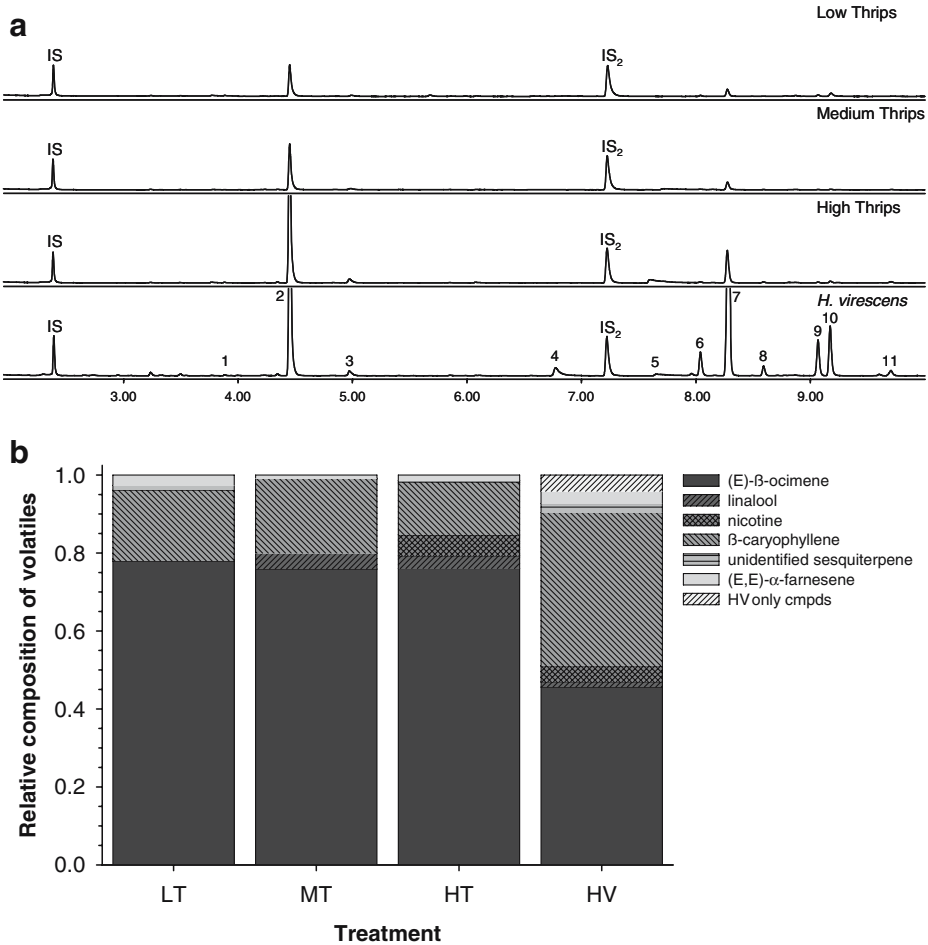


Fig. 1 Chromatograms showing typical daytime emission profiles (**a**) and composition (**b**) of volatiles released by tobacco plants receiving low (LT), medium (MT), and high levels of thrips feeding (HT) or *H. virescens* feeding (HV). Compounds are labeled as follows: *1*, myrcene; *2*, (*E*)-β-ocimene; *3*, linalool; *4*, indole; *5*, nicotine; *6*, β-elemene; *7*, β-caryophyllene; *8*, α-humulene; *9*, unidentified sesquiterpene; *10*, (*E, E*)-α-farnesene; *11*, caryophyllene oxide; *IS* and IS_2, internal standards (*n*-octane and *n*-nonyl-acetate)

Table 1 Amounts (nanogram per day; mean ± SE) of volatiles released on d 4 by tobacco plants receiving varying treatments

Compound	Treatment		
	LT	MT	HT
(*E*)-β-Ocimene	433±259a	494±114a	1100±141a
Linalool	0±0a	26.5±14.2a	46.9±5.78b
Nicotine	0±0a	0±0a	78±50.4a
β-Caryophyllene	100.9±62.8a	125.3±29.3a	195.9±54.4a
Unidentified sesquiterpene	6.8±6.8a	0±0a	5.9±5.9a
(*E,E*)-α-Farnesene	14.7±14.7a	5.11±5.11a	20.4±13.5a
Total volatiles	555±342a	651±157a	1447±232a

Numbers in each row followed by the same letter indicate no significant differences (Tukey's HSD test, $P<0.05$) Low levels of thrips feeding damage (LT), medium levels of thrips feeding damage (MT), and high levels of thrips feeding damage (HT) ($N=3$).

compounds (an unidentified sesquiterpene and (*E*,*E*)-α-farnesene) were also emitted in response to low levels of thrips feeding, but were only observed in one replicate (Table 1). In response to medium and high levels of thrips feeding damage, (*E*)-β-ocimene and β-caryophyllene remained the dominant compounds and were released in increasing amounts, although not significantly so ($P>0.05$; Table 1). In addition to releasing (*E*)-β-ocimene and β-caryophyllene, plants responded to medium and high levels of thrips feeding damage by releasing linalool (Table 1). Significantly more linalool was released by plants receiving high levels of thrips feeding damage compared to those with low levels of thrips damage (ANOVA: $F_{2,\ 7}=9.55$, $P=0.010$; Table 1). Nicotine was also released by plants in response to high levels of thrips damage, although the difference was not significant due to large variance (ANOVA: $F_{2,\ 7}=2.93$, $P=0.119$; Table 1). The compound (*E*,*E*)-α-farnesene was released in response to all three levels of thrips feeding damage, but it was only consistently released by plants receiving high levels of thrips damage (Table 1).

The highest level of thrips feeding induced the consistent release of five compounds (Table 1). In comparison, feeding by *H. virescens* induced the release of 11 compounds (Fig. 2; Table 2). Of the five shared compounds released by plants in response to thrips and caterpillar feeding, (*E*)-β-ocimene, linalool, β-caryophyllene, and (*E*,*E*)-α-farnesene were all released in greater amounts in response to caterpillar feeding ($P<0.05$). Because several of the compounds induced by thrips feeding were also among the most dominant compounds released in response to caterpillar feeding, it is conceivable that the observed differences are due solely to the differences in feeding damage. Additionally, because the feeding habits of thrips and caterpillars are so dissimilar, standardizing the amount of damage among treatments is problematic. However, we did observe consistent differences in plant volatile responses to caterpillars vs thrips that persisted over a range of thrips damage levels. Moreover, when we look at the relative proportions of individual compounds in each of the blends, we see that the ratios of (*E*)-β-ocimene to β-caryophyllene (i.e., the two dominant compounds released in response to thrips and caterpillars) are dissimilar (Fig. 1b). The relative proportion of (*E*)-β-ocimene to β-caryophyllene in the caterpillar blend is almost 1:1 (46 to 39%) vs 4.3:1 (78 to 18%), 4:1 (76 to 19%), and 5.8:1 (76 to 13%) in the low, medium, and high thrips feeding blends, respectively, which suggests that plants are responding differently to the two herbivores irrespective of damage (Fig. 1b).

Conceivably, these differences might still reflect differences in damage levels, as the observable leaf damage caused by caterpillar feeding is considerably more extensive than

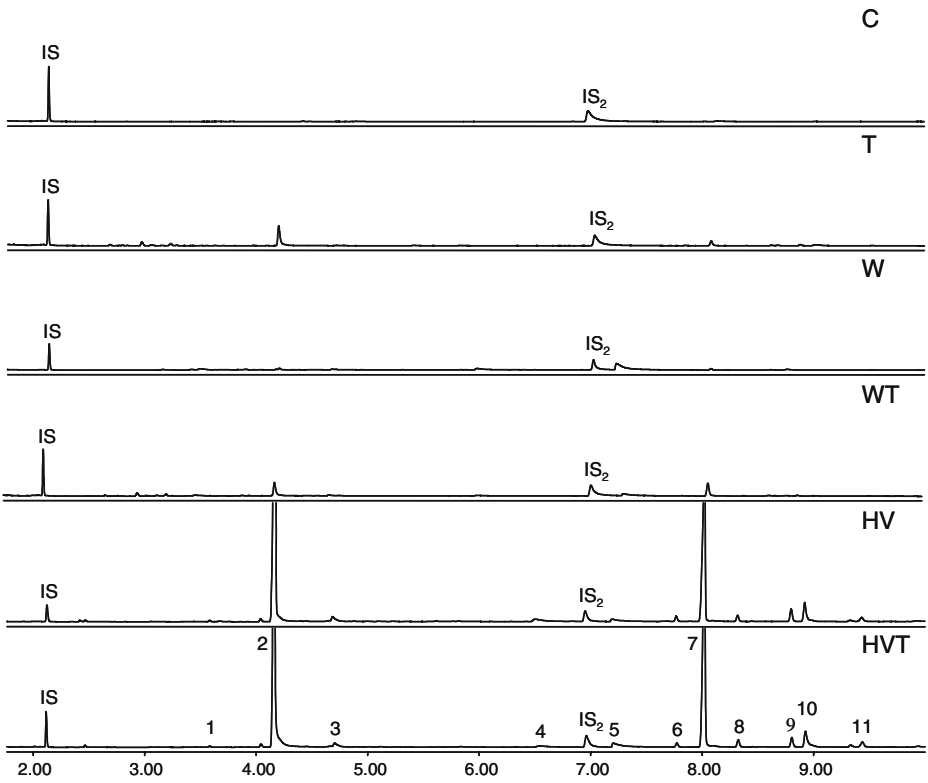


Fig. 2 Chromatograms showing typical daytime emission profiles of volatiles released by tobacco plants receiving the following treatments: unwounded control plants (C), thrips feeding (T), mechanical wounding (W), mechanical wounding plus thrips feeding (WT), *H. virescens* feeding (HV), or *H. virescens* plus thrips feeding (HVT). Compounds are labeled as follows: *1*, myrcene; *2*, (*E*)-β-ocimene; *3*, linalool; *4*, indole; *5*, nicotine; *6*, β-elemene; *7*, β-caryophyllene; *8*, α-humulene; *9*, unidentified sesquiterpene; *10*, (*E,E*)-α-farnesene; *11*, caryophyllene oxide; *IS* and *IS*$_2$, internal standards (*n*-octane and *n*-nonyl-acetate)

that caused by even high levels of thrips feeding ($P<0.001$), although leaf loss is probably a poor indicator of the damage inflicted by thrips feeding. Another approach to standardizing the treatments is to compare overall responses at damage levels where the amounts of the dominant compounds are similar. The level of (*E*)-β-ocimene emitted by plants on the second day of caterpillar feeding (data not shown) was similar to that released in response to high levels of thrips damage on d 4 ($P>0.05$; Table 1). Yet, the overall blends were notably different: all 11 compounds typically released in response to caterpillar feeding were induced by d 2 compared to only five compounds released in response to high levels of thrips feeding. Of the shared compounds, β-caryophyllene and (*E*,*E*)-α-farnesene were released in greater amounts in response to caterpillar feeding ($P<0.05$). There were no significant differences in the amounts of linalool or nicotine released by plants that were damaged by thrips or caterpillars ($P>0.05$). These results suggest that there are differences in plant responses to thrips and caterpillar feeding that cannot be entirely explained by differences in leaf-damage area.

Plants that were mechanically wounded or those that received mechanical wounding plus thrips feeding released three compounds [(*E*)-β-ocimene, nicotine, and β-caryophyllene;

Table 2 Amounts (nanogram per day; mean±SE) of volatiles released on d 4 by tobacco plants receiving varying treatments

Compound	Treatment					
	C	T	W	WT	HV	HVT
Myrcene	0±0a	0±0a	0±0a	0±0a	17.2±6.1b	22.25±3.3b
(*E*)-β-Ocimene	0±0a	433±259b	15.2±10.1a	121.1±44.7b	4,299±982c	5,315±1100c
Linalool	0±0a	0±0a	0±0a	0±0a	125.1±29.6b	178.3±34.3b
Indole	0±0a	0±0a	0±0a	0±0a	74.1±52.4b	141.7±66.8b
Nicotine	0±0a	0±0a	233±97.1b	160.1±76.4b	390±201b	538±217b
β-Elemene	0±0a	0±0a	0±0a	0±0a	89.6±45.2b	101.5±35.1b
β-Caryophyllene	0±0a	100.9±62.8b	39.5±8.5b	124.3±33.3b	3,704±684c	6,166±964c
α-Humulene	0±0a	0±0a	0±0a	0±0a	122.8±23.6b	208.6±34.7c
Unidentified sesquiterpene	0±0a	6.8±6.8a	0±0a	0±0a	219.2±87.7b	267.8±93.8b
(*E,E*)-α-Farnesene	0±0a	14.7±14.7a	0±0a	0±0a	293±106b	457±171b
Caryophyllene oxide	0±0a	0±0a	0±0a	0±0a	89.4±14.1b	166.3±33.6c
Total volatiles	0±0a	555±342b	288.2±94.9b	405.5±90.1b	9,423±2006c	13,562±1978c

Numbers in each row followed by the same letter indicate no significant differences (Tukey's HSD test on log-transformed data, $P<0.05$).

Unwounded control plants (C), thrips feeding (T), mechanical wounding (W), mechanical wounding plus thrips feeding (WT), *H. virescens* feeding (HV), or *H. virescens* plus thrips feeding (HVT) ($N=4$).

Table 2]. Nicotine was the most abundant compound released by mechanically wounded plants and by mechanically wounded plants that also received thrips feeding (Table 2).

Simultaneous Herbivory

Simultaneous feeding by thrips and *H. virescens* induced the release of the same 11 compounds emitted in response to *H. virescens* feeding alone (Fig. 2; Table 2). However, simultaneous herbivory induced the release of more α-humulene ($P<0.05$) and caryophyllene oxide ($P<0.01$; Table 2). There were no significant differences in the amount of leaf area removed by simultaneous herbivory (1,678±348 mm^2 damage/day) compared to caterpillar feeding alone (1,691±417 mm^2 damage/day), although this measure likely fails to capture the intensity of thrips feeding damage.

Thrips Distribution and Ovipositional Preferences

Effects of Mechanical Wounding

More adult thrips were found on unwounded plants (22.3±2.8 thrips/plant) than wounded plants (4.6±0.7 thrips/plant) over four sampling days (ANOVA: $F_{1,\ 40}=102.12$, $P<0.001$; Fig. 3). Thrips also displayed an apparent ovipositional preference for unwounded plants, with more larvae recovered on control plants (45±11.7 larvae/plant) compared to wounded plants (6.5±2.5 larvae/plant; $P<0.01$).

Effects of Caterpillar Feeding and Oral Secretions

We observed more adult thrips on unwounded plants than on any other treatment over six sampling days (ANOVA: $F_{4,\ 165}=15.74$, $P<0.001$; Fig. 4a). Additionally, more thrips were

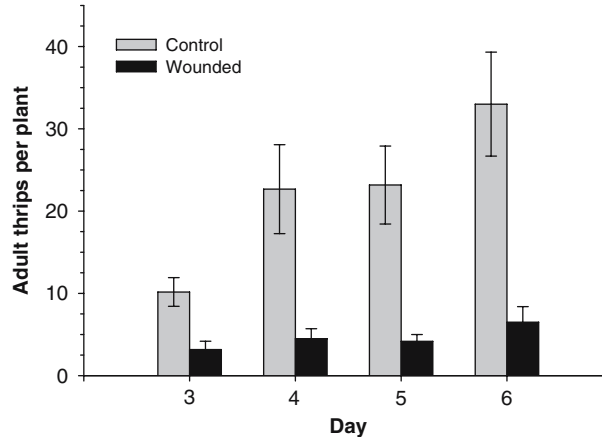


Fig. 3 Mean (±SE) number of adult WFT on control and mechanically wounded plants over four sampling days (N=6)

found on plants damaged by *H. virescens* and plants treated with *H. virescens* saliva than on plants treated with *H. virescens* regurgitant (P<0.01; Fig. 4a). Female thrips preferred to oviposit into unwounded control plants compared to mechanically wounded plants or plants treated with *H. virescens* regurgitant (P<0.05; Fig. 4b). Although there were no statistical differences, thrips appeared to prefer control plants as oviposition sites compared to plants damaged by *H. virescens* and plants treated with *H. virescens* saliva (Fig. 4b). They also appeared to prefer plants damaged by *H. virescens* and plants treated with *H. virescens* saliva compared to mechanically wounded plants and plants treated with *H. virescens* regurgitant, although, again, the observed differences were not statistically significant (Fig. 4b).

Effects of Volatile Nicotine

More adult thrips were found on plants encircled by blank (control) septa (19.78±3.34 thrips/plant) than on plants encircled by septa releasing nicotine (7.56±1.42 thrips/plant) over three sampling days (ANOVA: $F_{1,\ 14}$=33.3, P<0.001; Fig. 5). More larvae were also recovered on plants encircled by control septa (97.7±17 larvae/plant) compared to plants encircled by the nicotine septa (37±8.08 larvae/plant; P=0.032).

Discussion

Induced Plant Volatiles

Simultaneous feeding by multiple herbivores is common in natural ecosystems (Strauss 1991; Vos et al. 2001). Previous research has demonstrated that plant responses to feeding by a single herbivore species differ from response to attack by multiple herbivores (e.g., Shiojiri et al. 2001; Rodriguez-Saona et al. 2003) and that attack by multiple species can have both positive and negative effects on preference and performance of subsequent herbivores and herbivore natural enemies (Cardoza et al. 2002; Shiojiri et al. 2002; Rodriguez-Saona et al. 2005). In our study, simultaneous herbivory by thrips and *H. virescens* induced the same 11 compounds as caterpillar feeding alone, although there were significant differences in the relative amounts of two individual compounds, α-humulene and caryophyllene oxide. Total

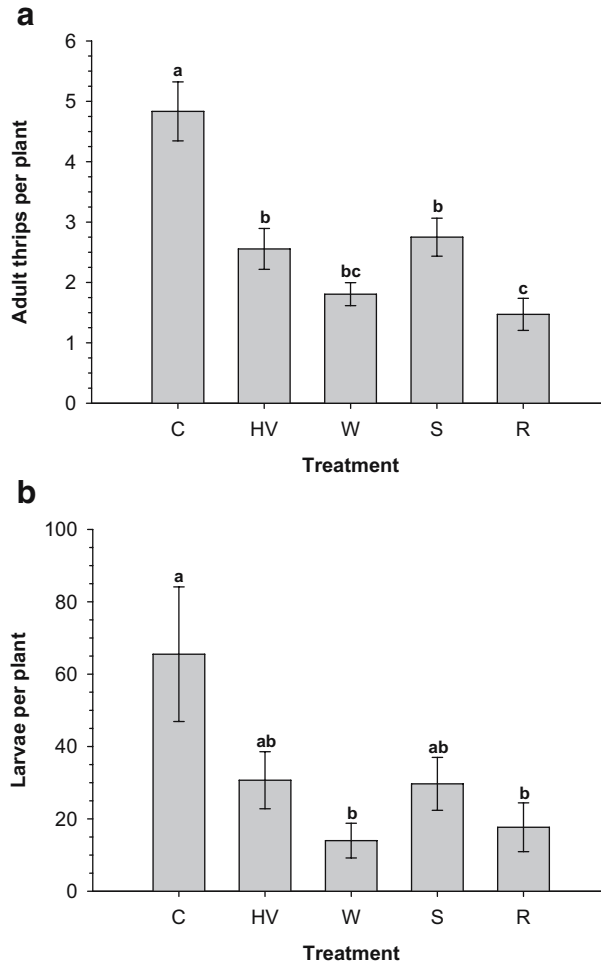


Fig. 4 Mean (±SE) number of adult WFT (**a**) and larvae (**b**) on plants receiving the following treatments: unwounded control plants (C), *H. virescens* feeding (HV), mechanical wounding (W), mechanical wounding plus *H. virescens* saliva (S), or mechanical wounding plus *H. virescens* regurgitant (R) (N=6). Letters above each bar indicate the significant differences as indicated by Tukey's HSD test, $P<0.05$

volatile production in our study was not reduced in response to simultaneous herbivory, in contrast to the 60% reduction observed in cotton plants when exposed to simultaneous feeding by silverleaf whitefly and beet armyworm compared to beet armyworm feeding alone (Rodriguez-Saona et al. 2003). However, our findings are similar to those reported by Cardoza et al. (2002) who found that simultaneous pathogen infection and herbivore feeding in peanut plants, *Arachis hypogaea*, induced the release of some compounds in higher quantities compared to herbivore feeding alone. Dual-damaged plants were also shown to be more attractive to the parasitoid *Cotesia marginiventris* (Cardoza et al. 2003). Similarly, Shiojiri et al. (2002) demonstrated an increase in parasitism by *Cotesia glomerata* on plants infested by two caterpillar species compared to plants infested with their host caterpillar alone. It remains to be determined whether the changes in volatile emissions that we observed in response to simultaneous infestation by thrips and caterpillars similarly alter the behavior of herbivore natural enemies.

Our results provide the first direct evidence that thrips induce volatiles and support previous research that provided evidence for the use of thrips-induced plant volatiles in

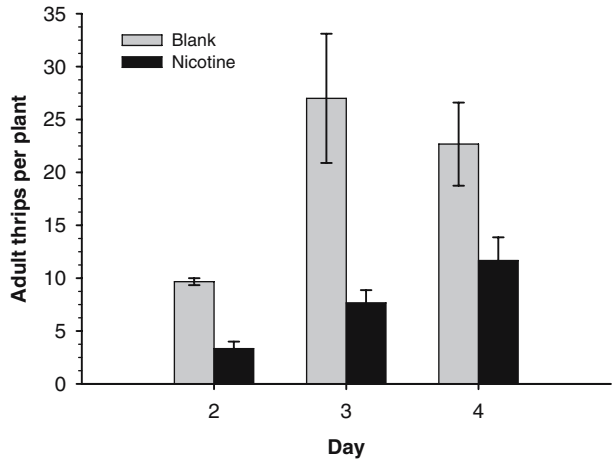


Fig. 5 Mean (±SE) number of adult WFT on unwounded plants encircled by blank rubber septa (control) and rubber septa containing nicotine over three sampling days (N=3)

both avoidance (Pallini et al. 1997) and attraction (Venzon et al. 1999) of heterospecifics to thrips-infested plants. Plants responded to feeding by the piercing–sucking insect WFT by emitting a volatile blend that was qualitatively and quantitatively distinct from that released in response to caterpillar feeding. While the very different feeding modes of these two herbivores make it difficult to standardize damage levels across treatments, the observed differences in volatile blends persisted across a wide range of thrips damage levels. Moreover, the overall volatile responses to thrips vs caterpillar feeding were different at damage levels where the amount of the most dominant compound released was similar. These results suggest that there are systematic differences in the volatile response to thrips vs caterpillar feeding that cannot be wholly explained by differences in the amount of damage inflicted. This contrasts with the previous finding by Turlings et al. (1998) that feeding by the stemborer *Ostrinia nubilalis* on maize induced a volatile blend qualitatively similar to that induced by caterpillar feeding but released in much lower quantities. The phloem-feeding aphid *Rhopalosiphum maidis*, which causes limited cell damage, did not induce measurable volatile emissions at all, although other aphid species elicit plant volatile responses (Du et al. 1996, 1998).

Thrips Distribution and Ovipositional Preferences

The ability of herbivores to distinguish among plants as suitable oviposition sites is important for the fitness of their offspring (e.g., Ohsaki and Sato 1994). Our results indicate that thrips are able to distinguish among plants as suitable hosts and that induced plant responses influence this preference. Thrips exhibited a consistent distributional preference for unwounded plants over all other treatments and preferred plants fed on by *H. virescens* and those treated with *H. virescens* saliva to those treated with *H. virescens* regurgitant (Figs. 3 and 4a). A similar pattern was observed for thrips ovipositional preferences, although significant differences were observed only between unwounded controls and plants that were mechanically wounded or treated with regurgitant (Fig. 4b). However, the fact that control plants had double the number of larvae compared to plants damaged by *H. virescens* and those treated with saliva suggests that adult thrips may be able to distinguish among these treatments as suitable oviposition sites for larval development

(Fig. 4b). While it is possible that poor egg hatch or increased larval mortality may have contributed to the low numbers of larvae recovered on wounded plants, we did not observe any dead adults or larvae throughout the choice experiments. Moreover, Maris et al. (2004) reported a direct correlation between the number of eggs laid and the larvae that emerged even on a resistant pepper variety.

The observed thrips preference for unwounded plants is consistent with the observation that the application of jasmonic acid, a natural elicitor of plant responses similar to those elicited by chewing herbivores, resulted in a decrease in the abundance of thrips on tomato (Thaler et al. 2001). Herbivore-induced plant responses were also found to reduce thrips feeding compared to un-induced control plants in the absence of prey (Agrawal et al. 1999). In contrast, Rodriguez-Saona and Thaler (2005) investigated the effects of herbivore-induced responses on the abundance of arthropods on tomato and found that patch type (i.e., induced vs un-induced) did not affect the abundance of thrips. However, because thrips are omnivores, prey availability may explain why patch type did not affect abundance (Agrawal et al. 1999).

We previously reported that tobacco plants treated with *H. virescens* regurgitant release significantly higher levels of volatile nicotine compared to those treated with *H. virescens* saliva or induced by caterpillar feeding (Delphia et al. 2006). Thus, we suspected that the similar pattern of thrips distribution and ovipositional preferences observed in this study might be explained, in part, by differential nicotine induction in plants treated with *H. virescens* saliva and regurgitant. In our choice assays, thrips preferred plants encircled by blank (control) septa compared to those encircled by septa releasing nicotine (Fig. 5), and more larvae were also found on the control plants, suggesting that nicotine has a significant effect on thrips behavior. This finding is consistent with studies demonstrating that induced plant volatiles can repel herbivores, such as ovipositing moths and aphids (Bernasconi et al. 1998; De Moraes et al. 2001).

Other authors have previously suggested a role for caterpillar saliva in suppressing induced plant defense mechanisms (Musser et al. 2002; Na and Chenzhu 2004; Bede et al. 2006). For example, glucose oxidase, an enzyme first identified from the saliva of the corn earworm, *Helicoverpa zea*, and subsequently found in other caterpillar species, suppresses the wound-inducible production of foliar nicotine in tobacco, *N. tabacum* (Musser et al. 2002). It is possible that foliar nicotine, which increases upon mechanical wounding (McCloud and Baldwin 1997; Musser et al. 2002), as well as other non-volatile metabolites (Duffey and Stout 1996), likely contributed to thrips distribution and ovipositional preferences observed in our experiments.

In summary, our results offer insights into plant responses to feeding by piercing–sucking insects, simultaneous feeding by herbivores with different feeding habits, and the influence of induced plant responses on subsequent herbivore behavior. Tobacco plants responded to all three levels of thrips feeding damage by releasing a volatile blend distinct from that released in response to caterpillar feeding. Simultaneous feeding induced the release of the same 11 compounds as caterpillar feeding alone; however, two compounds were produced in greater quantities. Induction of plant defenses had significant impacts on the distribution and ovipositional preferences of WFT: significantly more adults were found on unwounded control plants in both thrips choice tests, and more thrips were found on plants damaged by caterpillars and plants treated with caterpillar saliva compared to plants treated with caterpillar regurgitant. Furthermore, thrips preferred to oviposit into unwounded control vs wounded plants or plants treated with caterpillar regurgitant. These differences may be explained, at least in part, by the apparent repellant effect of nicotine, as significantly fewer adult and larval thrips were found on plants encircled by septa releasing volatile nicotine compared to those encircled by blank septa.

Acknowledgments We thank Anurag A. Agrawal, John F. Tooker, and James H. Tumlinson for helpful comments on the manuscript. We thank J. Saunders for logistical support and E. Bogus for technical assistance. The project was supported by the USDA National Research Initiative (#2002-35302-12375), the David and Lucile Packard Foundation, and the Beckman Foundation.

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J Chem Ecol (2007) 33:1013–1023
DOI 10.1007/s10886-007-9271-8

Foliar Phenolics are Differently Associated with *Epirrita autumnata* Growth and Immunocompetence

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Received: 25 October 2006 / Revised: 8 January 2007 / Accepted: 21 February 2007 /
Published online: 6 April 2007
© Springer Science + Business Media, LLC 2007

Abstract The quality of available food may affect insect herbivores directly (via growth and survivorship) and/or indirectly (by modifying insect vulnerability to parasitoids and pathogens). We examined the relationship between different phenolic compounds, belonging to various phenolic groups, in *Betula pubescens* spp. *czerepanovii* (mountain birch) foliage and the larval performance of the geometrid *Epirrita autumnata* (autumnal moth). Direct effects on insect performance were described by pupal weight, developmental rate, and survivorship; indirect effects were described by the encapsulation rate of an implant inserted into the insect hemocoel, a commonly used way to describe insect immune defense. We found profound differences in the effects of different phenolic categories: several individual hydrolyzable tannins were associated positively with larval performance but negatively with level of immune defense, whereas flavonoid glycosides were inversely related to larval survival but showed no association with the larvae immune defense.

Keywords Secondary compounds · Tritrophic interactions · Direct and indirect defense · Immunocompetence · Encapsulation

Introduction

Since the classic paper by Feeny (1970), many analyses of the chemical defenses of woody plants have focused on phenolic compounds. The results have been variable; correlations between individual phenolic compounds or groups of compounds and insect performance have often ranged from negative to zero, but positive correlations have also been reported (Karban and Baldwin 1997). Although it is possible, in a single study, to find negative correlations between individual phenolic compounds and insect performance, extensive

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studies of foliar chemistry in *Betula pubescens* spp. *czerepanovii* (Orlova) Hämet-Ahti (mountain birch), have not identified any particular compound that would consistently explain the performance of herbivorous insects (reviewed in Haukioja 2003).

This failure to identify defense mechanisms contrasts strongly with the situation in laboratory studies, often using *Arabidopsis* or agricultural plants. These studies have established sophisticated general defense cascades (Karban and Baldwin 1997), which often utilize indirect defenses, i.e., recruiting parasitoids and predators to attack the herbivores (Havill and Raffa 2000), thus protecting the plant (Agrawal 1999).

Outbreaks of herbivorous insects are characteristic of many ecosystems. If the defense mechanisms of a plant play a role in the population dynamics of its herbivores, then the level of defenses should increase at and after peak herbivore density. Attempts to demonstrate this have given indications that the defenses of woody plants indeed function in a delayed density-dependent way (Benz 1974; Kaitaniemi et al. 1998). There is, however, no consensus as to the importance of plant defenses in the population dynamics of the herbivore. Uncertainties as to which plant traits should be measured have made it difficult to find out whether phenolic compounds, or plant defenses in general, are a factor contributing to or driving herbivore population outbreaks and cycles (Haukioja 2005a, b).

Another reason for a skeptical attitude toward the importance of induced plant defenses behind defoliator outbreaks and cycles is that time series models have repeatedly shown that parasitism seems to be sufficient to explain declines in population density (see examples in Berryman 2002). These analyses search for factors with delayed, negative density dependence, implicitly suggesting that the increase phase does not demand any explanation at the chemical level. Kapari et al. (2006), however, have demonstrated that during the early increase phase of the *Epirrita autumnata* (Bkt.) Lep., Geometridae (autumnal moth) cycle, the immune defense of this natural and important defoliator of the *B. pubescens* varies among individual host trees. Interestingly, the index of immune defense was higher in trees with artificially introduced larvae in the previous year than in control trees, many of which still were devoid of larvae. Because the increase phase of the *E. autumnata* cycle is characterized by a low level of parasitism (Klemola et al. 2004), this result suggests an unanticipated connection between induced changes in plant defense and the resistance of defoliators toward their enemies (parasitoids and diseases). It also implies that defoliator outbreaks and cyclic peaks may be facilitated, perhaps even caused, if defoliators benefit from the compounds produced by the plants against them (Haukioja 2005b).

The effects of host plant chemistry in tritrophic interactions have been studied mainly in herbaceous plants and in insect–pathogen systems (reviewed in Cory and Hoover 2006). Often, individual phenolic compounds in food inhibit viral infection (Felton et al. 1987; Keating et al. 1990; Young et al. 1995; Hoover et al. 1998) but not always (Roth et al. 1997; Lindroth et al. 1999). Chlorogenic acid in the diet increases the toxicity of bacteria (Ludlum et al. 1991). Some alkaloids have antifungal effects (Gallardo et al. 1990).

The diet of an herbivore also affects parasitoids (e.g., in *Populus*; Auerbach and Alberts 1992; Holton et al. 2003; Parry et al. 2003), and the effects of individual secondary chemicals on parasitoids are potentially important (Ode et al. 2004; Ojala et al. 2005). Different food-quality-dependent defense mechanisms against predators are common in herbivorous insects: sawflies, for instance, are better at protecting themselves against ants if the food plant contains abundant resin acids (Bjorkman and Larsson 1991), and *Chrysomelidae* larvae are able to secrete a repellent, which contains compounds obtained from the food plant (Hilker and Schulz 1994). In woody plants, the high levels of foliar hydrolyzable tannins made *Lymantria dispar* larvae more resistant against their viral diseases (Hunter and Schultz 1993; Keating et al. 1988, 1990; but see also Lindroth et al.

1999). In contrast, phenolic glycoside salicin increased *L. dispar* larval susceptibility to viral infection (Cook et al. 2003).

To determine how secondary chemicals directly and indirectly affect an insect defoliator, we analyzed ca. 50 phenolic compounds of birch foliage in the early phase of the natural population cycle of the geometrid *E. autumnata*. The compounds covered the main phenolic groups: condensed tannins (proanthocyanidins), hydrolyzable tannins (including ellagitannins and gallotannins [galloylglucoses]), *p*-coumaroylquinic acid derivatives, and flavonoid glycosides. We tested whether phenolics correlate with the indirect and direct resistance of *B. pubescens* during the early increase phase of a defoliator population. We correlated the contents of phenolic compounds with larval survivorship, pupal weight, and duration of the larval period (to detect associations relating to direct defenses), and with insect immune defense, measured as the encapsulation rate (to detect associations relating to indirect defenses). The main results demonstrate that several individual hydrolyzable tannins are associated positively with larval performance but at the same time negatively with level of immune defense.

Methods and Materials

Species Betula pubescens spp. *czerepanovii* (mountain birch) is the dominant tree in Northern Finland, and forms the tree line in NW Europe (Kallio and Mäkinen 1978). The most harmful herbivore of is *Epirrita autumnata*, which has cyclic large-scale outbreaks (Tenow 1972; Ruohomäki et al. 2000). *E. autumnata* has several species of parasitoids (Ruohomäki 1994; Ruohomäki et al. 2000) and pathogens (Bylund 1995).

Setup In the summer of 2002, 100 mature mountain birches were randomly selected from a natural population near the Kevo Subarctic Research Station (Northern Finland, 69°45′N, 27°01′E), as described in Kapari et al. (2006). The birches were mature with at least three ramets (stems). During that field season, we reared *E. autumnata* larvae in half of the trees. Larvae were reared in two mesh bags on different branches of each tree. Control trees had empty mesh bags attached. During the next field season (2003), 60 of these 100 trees (30 control and 30 larval trees) were randomly selected for the experiment.

The experiment was conducted in 2003 by attaching one mesh bag to a branch on the same trunk that was bagged the year before. The branches selected were different from those bagged in 2002. Each bag contained at least 150 leaves (at least 50 short shoots). At the time of leaf flush at the end of May, 10 (in 2002) or 15 (in 2003), newly hatched moth larvae were released into each bag: 20 larvae per tree in 2002, and 15 in 2003. Each bag contained an identical mixture of the *E. autumnata* broods. At the end of the larval period, the bags were checked daily. Larvae were collected when they started to pupate and were placed individually in plastic vials embedded in moist moss. The duration of the larval period was recorded for each larva (N=374). After 7 d as a pupa, the moth pupa was weighed and sexed (N=368) and its encapsulation rate was measured (N=356).

The level of insect immune defense was estimated by a commonly used technique that measures the efficiency with which insect hemocytes encapsulate a foreign object in their hemocoel (see, e.g., Vainio et al. 2004; Rantala and Roff 2005). We inserted an implant (a nylon monofilament, 2 mm long, 0.20 mm in diameter, rubbed with sandpaper) into the hemocoel of 1-wk-old pupae. Pupae were incubated +14°C for 1 hr to allow encapsulation and then frozen in liquid nitrogen (the method is described in detail in Kapari et al. 2006). Later, the implant was removed and photographed from three different angles under a light

microscope with a digital video recorder. As a measure of encapsulation rate, we used the average gray values of three video pictures (the observed gray values were subtracted from the gray value of a clean implant (see details in Kapari et al. 2006). The ability to encapsulate abiotic material is related to the ability to encapsulate a parasite (Paskewitz and Riehle 1994; Gorman et al. 1996). Previous studies with the *E. autumnata* suggest that the encapsulation rate of nylon monofilament is associated with resistance against an entomopathogenic fungus (Rantala and Roff, unpublished data).

Analyses To determine the chemical properties of *B. pubescens*, we measured the concentrations of condensed tannins (proanthocyanidins), 24 hydrolyzable tannins (12 gallotannins [galloylglucoses] and 12 ellagitannins), 15 flavonoid glycosides, 4 *p*-coumaroylquinic acid derivates, and 2 catechins from the leaves (Table 1).

A total of 6–10 leaves from short shoots were sampled randomly throughout the canopy of the experimental trees (within 2 hr on the morning of June 27 2003) when larvae were on average at the beginning of their last (fifth) instar. Leaves were sealed in plastic bags and transported to the laboratory in a cooler; they were weighed and freeze-dried (72 hr). Dried leaves were reweighed, and their initial water content was determined from the fresh and dry weight of the samples. Dry leaf samples from each tree were then homogenized to a powder and stored in plastic vials at −20°C.

A sample of the birch leaf powder (30.0±1.0 mg) was placed in a plastic 1.5-ml Eppendorf tube, and 0.65 ml of 80% aqueous acetone were added. The sample was allowed to stand for 30 min at room temperature with continuous stirring (1.5 U, Vortex, Genie 2). The homogenate was centrifuged for 10 min at 2,500×*g*, and the first portion of extract was transferred into a 2.0-ml plastic Eppendorf tube. The pellet was reextracted with 0.65 ml of 80% acetone, and then twice more with the same volume of 100% acetone. All extracts were combined, and the acetone was removed in a vacuum concentrator (Concentrator 5301, Eppendorf AG, Germany). The resulting aqueous phase was frozen and lyophilized for 24 hr. The insoluble residue, including proteins, starch, cellulose, lignin, etc., was dried under a vacuum for 24 hr and weighed, and the proportion of acetone-insoluble compounds in the sample was determined.

The lyophilized extract was dissolved in 1.0 ml of the chloroform/methanol/water mixture (50/30/20, v/v/v) and vortexed for 25 min. Then 0.5 ml of 20% aqueous methanol was added. The forming biphasic system was vortexed for 10 min and centrifuged for 10 min at 2,900×*g* (+4°C) to separate the two solvent phases (chloroform/methanol and methanol/water). The methanol/water fraction of polar compounds was concentrated in a vacuum concentrator to remove methanol, and the water phase was frozen and lyophilized. The dry residue of polar compounds was dissolved in 0.7 ml of water, centrifuged for 20 min at 2,900×g, and stored at −20°C.

Individual phenolics in the fraction of polar compounds (0.1 ml) were studied by using the high-performance liquid chromatography (HPLC) diode-array detection system as in Salminen et al. (1999), except that the solvents used were (1) 0.05 M of H_3PO_4, and (2) acetonitrile. A selected set of samples was also analyzed with HPLC electrospray ionization mass spectrometry as in Salminen et al. (1999), except that the data system used was Analyst Software 1.1. Phenolics were identified on the basis of their UV, mass spectra, and retention times.

For the quantification of polar phenolics, the HPLC raw data of all samples were exported in COWTool software (COWTool version 1.1., Department of Biotechnology, Technical University of Denmark, Denmark) in text format and subjected to baseline correction to reduce the noise level. To correct retention time differences among different samples, the chromatograms derived from each sample were later aligned against the master

Table 1 Means and standard deviations of concentrations of compounds measured in *B. pubescens* spp. *czerepanovii* (mountain birch) leaves

Name of Compound	Mean	SD	Encapsulation	Weight	Development Rate	Survival
Flavan-3-ols						
Condensed tannins (proanthocyanidins)	98.39	22.73				−0.25
plus-Catechin	23.38	5.8		0.29		−0.36
Catechin derivate	2.96	1.49				
Gallotannins (hydrolyzable tannins)						
1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose	19.67	10.85		0.34		
1,2,3,6-tetra-*O*-galloyl-*β*-D-glucose	5.58	4.07				
1,2,6-tri-*O*-galloyl-*β*-D-glucose	28.59	19.93				
Digalloylglucose	24.42	15.27	−0.25		0.20	
1-*O*-galloyl-*β*-D-glucose	120.5	76.54	−0.29			
Tetragalloylglucose	37.94	26.99	−0.26			
Trigalloyl-HHDP-glucose	4.18	3.74				
Gallotannin 1	3.26	1.48				−0.29
Gallotannin 2	6.48	4.65	−0.31			
Gallic acid derivate 1	1.26	0.86	−0.35			
Gallic acid derivate 2	7.03	5.86				
Gallic acid derivate 2b	6.15	1.93		0.22	0.31	
Ellagitannins (hydrolyzable tannins)						
Pedunculagin	23.94	21.4			0.21	0.32
Pedunculagin derivative	44.34	32.04				
Tellimagrandin I	1.67	1.24				
Ellagitannin 1	1.90	0.60				
Ellagitannin 2	4.21	2.04				
Ellagitannin 3	10.62	4.57	−0.26			
Ellagitannin 4	7.34	10.32			0.19	
Ellagitannin 5	31.32	21.37			0.24	
Ellagitannin 6	2.56	1.43				
Ellagitannin 7	6.41	3.84			0.28	
Ellagitannin 8	9.02	7.98	−0.22		0.19	
Ellagitannin 9	2.71	2.58	−0.24			
Flavonoid glycosides						
Myricetin-3-*O*-*β*-D-galactopyranoside	22.11	12.61				
Myricetin-3-*O*-*β*-D-glucuronopyranoside	7.63	7.65				−0.22
Kaempferol-3-*O*-a-L-rhamnopyranoside	16.09	10.12				
Kaempferol-3-*O*-*β*-D-acetyl-glucopyranoside	5.45	10.91				
Kaempferol-3-*O*-*β*-D-glucopyranoside	11.11	5.73				
Kaempferol-3-*O*-*β*-D-glucuronopyranoside	22.46	12.66				
Kaempferol glycoside 1	0.94	1.28				−0.27
Kaempferol glycoside 2	0.31	0.29				
Quercetin-3-*O*-a-L-acetyl-glucopyranoside	2.83	4.40				
Quercetin-3-*O*-a-L-arabinofuranoside	4.85	6.84				

Table 1 (continued)

Name of Compound	Mean	SD	Encapsulation	Weight	Development Rate	Survival
Quercetin-3-*O*-*β*-D-galactopyranoside	54.38	32.72			0.26	
Quercetin-3-*O*-*β*-D-glucuronopyranoside	98.07	36.52				
Quercetin glycoside 1	1.64	1.26				
Quercetin glycoside 2	21.88	10.72				
Quercetin glycoside 3	14.88	24.32				
p-Coumaroylquinic acid derivatives						
Chlorogenic acid	249.51	93.64				
Neochlorogenic acid	6.27	12.32				
trans-3-*p*-Coumaroylquinic acid	28.18	14.09		0.20		
trans-5-*p*-Coumaroylquinic acid	3.83	5.14				

Means and standard deviations were estimated with bootstrap. The correlations between secondary compounds of birch and larval traits (encapsulation rate, pupal weight, and larval development rate, and survival) were calculated with bootstrap. Only those bias-corrected correlation coefficients are shown whose 95% confidence limits do not include zero. Content of low molecular mass phenolics is expressed as a peak height per 1 g of dry mass; condensed tannin content is measured as milligram per 1 g of dry mass.

HHDP = Hexahydroxydiphenoyl

chromatogram of polar phenolics. The processed raw data were exported in Excel software and used to determine the retention times and heights of all phenolic peaks. The relative content of individual phenolics was calculated as peak height per 1 g of dry mass of the leaf sample.

To determine the condensed tannin (proanthocyanidin) content, 0.05–0.1 ml of the polar fraction was used (Ossipova et al. 2001).

Statistical Analyses Phenolic compounds used in the statistical analyses, with means and standard deviations of the concentrations, are shown in Table 1. Four compounds (1-6-di-galloyl-glucose, tetragalloylglucose, pedunculagin, and pedunculagin derivative) had two isomers. We present them as the sum of these two isomers, and used their combined content in statistical analysis.

Tree-specific least square means were calculated for each dependent variable (encapsulation rate, pupal weight, duration of larval period, and larval survival) with ANOVA. For encapsulation rate values (mean=47.6, SD=9.7) and for duration of the larval period (mean=44.8 d, SD=2.2), the tree-specific means were computed including sex as a random factor in the model. The effect of sex on pupal weight (mean=57.4 mg, SD=8.1) was removed by transforming male weights into female weights by using the formula given in Haukioja and Neuvonen (1985). In the original data, mean pupal (female) weight was 57.9 mg (N=368, SD=10.6), mean duration of the larval period was 44.2 d (N=374, SD=3.0), and the mean encapsulation rate was 46.0 (N=356, SD=18.1). Survival was measured as the number of larvae in one tree surviving until pupation. Survival percentages ranged from 0 to 93%, with a mean of 42% (SD=20) (Kapari et al. 2006).

Other things being equal, a short larval period in *E. autumnata* indicates good growth (Kause et al. 1999a, b). To help interpret growth rate, we constructed a new variable, the development rate, defined as the reciprocal of the duration of the larval period (in days). Thus, for each variable (pupal weight, survival rate, and developmental rate), a positive correlation with an individual compound indicates that larvae did well.

Correlations between tree-specific least square means and concentrations of phenolics were calculated as *Spearman's* nonparametric correlations with the PROC CORR (SAS Institute 1990), as the concentrations of compounds were not normally distributed. We used *bootstrap* analysis to estimate the 95% confidence limits for individual correlation coefficients (Dixon 2001). Bootstrap estimates the precision of the correlation coefficient by creating random resamples from the data. With bootstrap, the distribution of the data is better taken into account than by using *Bonferroni* correction. If zero was not included in the 95% confidence limit, we assumed that the correlation coefficient differed from zero. The 95% confidence limit in our data was on average ±0.24. In the bootstrap analysis, we used 200 resamples and the % boot macro. In Fig. 1, we marked every individual correlation into its biochemical group. Larval treatment of the previous year did not affect the concentrations of compounds. The treatment or the sex of the larvae did not significantly affect the correlation coefficients.

Results

The immune response measured by encapsulation rate correlated negatively with 8 (out of 24) individual hydrolyzable tannins in birch foliage (mean correlation coefficient −0.15).

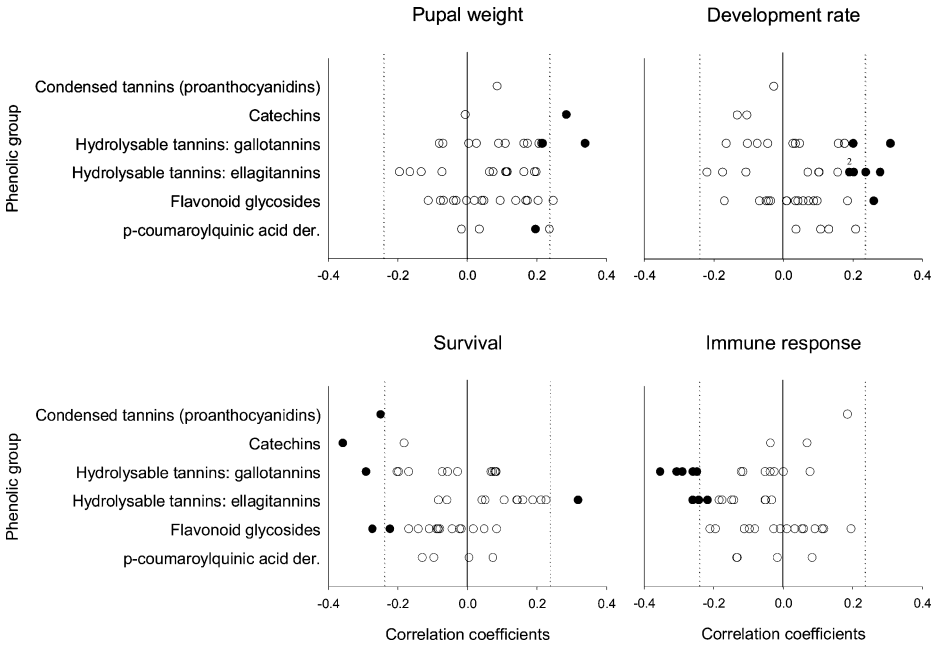


Fig. 1 Overview of correlations between *E. autumnata* (autumnal moth) traits and phenolic contents in *B. pubescens* spp. *czerepanovii* (mountain birch) foliage. The figure shows the correlation coefficients of individual phenolics (belonging to the condensed tannins [proanthocyanidins], catechins, hydrolyzable tannins [gallotannins and ellagitannins], flavonoid glycosides, or *p*-coumaroylquinic acid derivates) with *E. autumnata* traits (pupal weight, larval development rate, larval survival, and pupal encapsulation rate). Filled circles represent correlations significantly different from zero. A number 2 indicates two overlapping filled circles. The dotted line represents the mean 95% confidence limit around zero

Hydrolyzable tannins had mostly positive associations with the other moth traits. There was a positive correlation with pupal mass (0.08) and with larval development rate (0.08). In addition, the survival rate of the larvae was positively correlated with the contents of one type of hydrolyzable tannins, ellagitannins (0.12), but not with the other type, i.e., gallotannins (−0.05) (Fig. 1).

Condensed tannins had a negative association with larval survival (−0.23) but no relationship to larval encapsulation rate (0.19). The correlation coefficients between condensed tannins and pupal weight (0.09) and the larval development rate (−0.03) were close to zero, suggesting that condensed tannins had no direct impact on the growth of the *E. autumnata* (Fig. 1).

Flavonoid glycosides were generally negatively associated with larval survival (−0.08) and positively with pupal weight (0.06) and larval development rate (0.04). In contrast to hydrolyzable tannins, we did not detect any relationship between flavonoid glycosides and the immune response of the *E. autumnata* (−0.01) (Fig. 1).

Discussion

Within all phenolic groups, we found large variation in the correlations of individual compounds with moth traits. Some trends, however, were obvious, the most remarkable being the negative association between hydrolyzable tannins and the immune response measured as the melanotic encapsulation rate. We also noted that hydrolyzable tannins were positively related to pupal weight and larval development rate, and that flavonoid glycosides were negatively related to larval survival.

The phenolics compounds of *B. pubescens* spp. *czerepanovii* leaves often represent more than 20% of leaf dry weight, the bulk of the secondary compounds in leaves. There are dramatic shifts in the proportions of different phenolic groups during leaf growth and maturation. Flavonoid aglycones are a dominant phenolic group in bursting buds (up to 4% leaf dry mass), whereas young leaves are characterized by high levels of hydrolyzable tannins and flavonoid glycosides. Some flavonoid glycosides correlated negatively with larval mortality. In mature leaves, levels of hydrolyzable tannins tend to decrease or stay unchanged (Salminen et al. 2001) whereas levels of condensed tannins increase markedly, reaching in some trees concentrations as high as 15–20% of leaf dry mass (Riipi et al. 2002; Haukioja 2003). In addition, during leaf maturation water and protein concentrations decline and the leaves become tougher (Haukioja et al. 2002). The fact that the chemical composition of mountain birch changes dynamically during the season complicates the interpretation of the correlation results (Riipi et al. 2002). Fortunately, despite the massive changes in the foliar chemistry during the season, individual birches consistently have either a relatively low or high content of phenolics (Riipi et al. 2004).

The levels of condensed tannins, the most abundant foliar phenolics, were negatively related to survival rate of *E. autumnata* larvae. This was unexpected because condensed tannins are sometimes considered to be defenses of mature leaves (but see Ayres et al. 1997). There were no other associations between foliar condensed tannins and moth traits. The negative association between survival and condensed tannins, together with the positive correlations between hydrolyzable tannins and larval performance, may at least partly reflect the dependence of *E. autumnata* larvae on young foliage. However, because most *E. autumnata* mortality occurs during the first instars and we did our chemical sampling at the beginning of the fifth (last) instar, it is difficult to draw explicit conclusions from the correlation results.

Traditionally, hydrolyzable tannins have traditionally been considered to play an important part in a plant's direct resistance against herbivores. In our study, however, the higher the concentration of hydrolyzable tannins (both gallotannins and ellagitannins), the heavier the pupae and the more rapid the larval development. This counterintuitive result has been observed earlier (Kause et al. 1999b). In other studies, however, a negative relationship between hydrolyzable tannins and larval performance has been found (Kause et al. 1999a, Ossipov et al. 2001).

Intriguingly, the relationship of hydrolyzable tannins on the immune response measured as the encapsulation rate was the reverse of the relationship on direct resistance. A possible explanation is that insect melanotic encapsulation is a resource-demanding process and may therefore trade off with insect traits relating to growth (Rantala and Roff 2005). The tannin composition of the larval diet could affect moth investment in growth and immune response.

The negative correlation between immune response and hydrolyzable tannins is interesting for two reasons. First, the direction of the correlation is the opposite of what would be intuitively anticipated and what has been found in a different system, consisting of oaks and gypsy moth and their viruses (Hunter and Schultz 1993; Keating et al. 1988, 1990; but see also Lindroth et al. 1999). Second, unpublished results have demonstrated a significant increase in hydrolyzable tannins in birch foliage during the peak phase of the *E. autumnata* cycle. If the negative within-year correlation between immune response and hydrolyzable tannins, which we found, also holds in among-year comparisons, this would suggest that foliar chemistry promotes escape of the herbivore from its enemies during the increase phase of the population cycle (leaves still relatively low in hydrolyzable tannins), but no longer at later stages of the cycle (high hydrolyzable tannin content).

Acknowledgements We thank the staff of the Kevo Subartic Research Station for making this study possible. Irma Saloniemi and Kari Saikkonen gave helpful comments on the manuscript. Ellen Valle checked the language. The study was supported financially by the Academy of Finland: project 212712 (EH), 206144 (TR), 201073 (VO), and 207294 (MJR). The experiments presented comply with the current laws of Finland.

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J Chem Ecol (2007) 33:1025–1039
DOI 10.1007/s10886-007-9280-7

Effects of Enhanced UV-B Radiation on Plant Chemistry: Nutritional Consequences for a Specialist and Generalist Lagomorph

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Received: 10 September 2006 / Accepted: 2 March 2007 /
Published online: 4 April 2007
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Abstract Ultraviolet-B (UV-B) radiation has been increasing in temperate latitudes in recent decades and is expected to continue rising for some time. Enhanced UV-B radiation can change plant chemistry, yet the effects of these changes on mammalian herbivores are unknown. To examine the influence of enhanced UV-B radiation on nutrition of a specialist and generalist hindgut fermenter, we measured nutritional and chemical constituents of three common North American range plants, big sagebrush (*Artemisia tridentata*), yarrow (*Achillea millefolium*), and bluebunch wheatgrass (*Pseudoregneria spicata*), and how these changes influenced *in vitro* dry matter digestibility and *in vivo* digestibility by pygmy rabbits (*Brachylagus idahoensis*) and eastern cottontails (*Sylvilagus floridanus*). Forages were irradiated for 3 mo with ambient (1×) or supplemental (1.6×) UV-B radiation representing a 15% ozone depletion for Pullman, WA, USA. Enhanced UV-B radiation had minimal effects on the nutritional content and the tannin-binding capacity of forages. Similarly, the terpene concentration in sagebrush and yarrow was not affected by higher UV-B irradiances. Flavonoid compounds increased in sagebrush but decreased in yarrow. Rabbit preference and intake was not affected by treatment levels for any forage species and no differences were found between treatments for dry matter, fiber, protein digestibility, and apparent digestible energy.

Keywords Cottontail · Herbivore · Flavonoids · Phenolics · Pygmy rabbit · Secondary compounds · Terpenes · UV-B

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Introduction

Stratospheric ozone (O_3) depletion has led to enhanced surface ultraviolet-B (UV-B; 280–320 nm) radiation at temperate latitudes of both hemispheres in recent decades (McKenzie et al. 1999; UNEP/WMO 2002), and full recovery of the ozone column is not expected before 2050 (Anonymous 2000; Schrope 2000). Enhanced UV-B radiation can reduce growth and yield and alter photosynthesis in terrestrial plants (Reddy et al. 2004), change plant morphology, including height, leaf area, leaf waxes, and epidermal layers (Liakoura et al. 2003), and damage deoxyribonucleic acid (DNA) and photosystem II (PSII) (Keiller et al. 2003). However, the primary effect of enhanced UV-B radiation on plants seems to be changes in carbon partitioning from growth pools to secondary metabolic pathways (Tegelberg et al. 2002; Warren et al. 2002a, b; Bassman 2004).

Changes in plant secondary metabolites have the potential to influence herbivores that eat them, by reducing palatability, causing toxicity, or altering intake and digestibility by either inhibiting rumen bacteria or interfering with the animal's metabolism (Launchbaugh 1996). Behavioral, morphological, and physiological adaptations of herbivores influence their ability to avoid or tolerate plant secondary metabolites. Specialist herbivores are expected to have mechanisms for tolerating large amounts of chemicals produced by plants for which they are specifically adapted, whereas generalists are expected to avoid plant secondary metabolites or mix diets so as to acquire only small amounts of a variety of chemicals (Launchbaugh 1996; McCloud and Berenbaum 1999; Sorensen and Dearing 2003). The response of specialist and generalist herbivores to chemical components of plants altered by enhanced UV-B radiation has been studied in invertebrates (McCloud and Berenbaum 1999; Warren 2002a) but not in mammals.

In this study, we investigated the effects of enhanced UV-B radiation on secondary metabolites of three forage species common to northwestern rangelands (i.e., big sagebrush [*Artemisia tridentata*], yarrow [*Achillea millefolium*], and bluebunch wheatgrass [*Pseudoregneria spicata*]) and the nutritional response of two lagomorphs that occupy these rangelands. The pygmy rabbit (*Brachylagus idahoensis*) is a specialist feeder on terpene-containing sagebrush (Thines et al. 2004), whereas the eastern cottontail (*Sylvilagus floridanus*) is a generalist herbivore that eats a wide variety of grasses, forbs, and shrubs (Warren and Kirkpatrick 1978). Both were used previously in a nutritional diet choice study (Shipley et al. 2006). We expected that enhanced solar UV-B radiation would induce production of secondary plant metabolites and/or changes in nutritional quality that in turn would alter the palatability, intake, and digestibility of the food. Our objectives were to: (1) determine the effects of enhanced solar UV-B radiation on the concentration of predominant secondary compounds in sagebrush, yarrow, and bluebunch wheatgrass; (2) quantify the effects of enhanced UV-B radiation on nutritional quality of these same forage species; and (3) quantify the response for a specialist and generalist mammalian herbivore through preference and digestibility.

Materials and Methods

Plant Material and Growth Conditions The study was conducted both in a glasshouse and the in field at E. H. Steffen Center on the Washington State University campus in Pullman, WA, USA (46°44′N, 117°10′W, elevation 777 m) during the winter and summer of 2004. We selected a native shrub, forb, and grass to cover a range of nutritional qualities and secondary plant compounds. Big sagebrush (*Artemesia tridentata*) produces abundant

terpenes. This plant can comprise up to 98% of the diet of the pygmy rabbit, whereas the cottontail consumes only trace amounts (Thines et al. 2004). Bluebunch wheatgrass (*Pseudoregneria spicata*) contains few plant secondary compounds but has thick cell walls containing cellulose and lignin, and yarrow (*Achillea millefolium*) is a plant known also for its terpene and tannin content (Chandler et al. 1982).

We germinated unstratified seeds of sagebrush and yarrow in pots (Ray Leach container super cell, 164 cm^3, Stuewe and Sons, Corvallis OR, USA), and bluebunch wheatgrass in flats (50.8×35.6×8.9 cm) with a standard nursery-potting medium (Sunshine Mix #4, Sungro Horticulture, Bellevue, WA, USA). Plants were watered and fertilized with a commercial liquid fertilizer containing N, P, and K (20-20-20) and micronutrients. We extended the photoperiod in the glasshouse to 16 hr with high-pressure sodium, high-intensity discharge lamps (HPS/HID, 1000 W).

After seeds germinated, we exposed plants to two UV-B treatments supplied over an 8-hr period centered at solar noon and provided by Q-panel UV-B-313 fluorescent lamps (Q-Panel, Cleveland, OH, USA). Treatment levels included: 1) ambient (1×) biologically effective UV-B radiation at Pullman, WA on the summer solstice (7.5 kJ kJ^{-m} d^{-1}), or 2) 1.6× ambient supplemental UV-B radiation to simulate a 15% ozone depletion over Pullman, WA. We determined biologically effective UV-B radiation based on the new plant action spectrum of Flint and Caldwell (2003).

Sagebrush and yarrow were grown in the glasshouse during late winter and spring of 2004. Four UV-B lamps were mounted in frames (1.25×1.25 m) and suspended over plants. We adjusted the frame distance above the canopies to alter radiation levels by using an Optronics 754 spectroradiometer to measure UV-B radiation (Optronics Laboratories, Orlando, FL, USA). Lamps were filtered with 0.13 mm thick clear cellulose acetate film (transmission ≥292 nm) that were presolarized for 6 hr to stabilize transmission of filters. At 1-wk intervals, we replaced filters, rotated plants around the glasshouse to minimize location effects, and adjusted lamp height to maintain correct UV-B treatment levels.

We also grew bluebunch wheatgrass in flats banked with local soil to maintain root temperatures in an open field adjacent to the glasshouse during the summer of 2004. The same two treatments were examined. In the field, treatments were supplied by a stepped square-wave lamp system with eight UV-B lamps mounted in 1.2×2.4 m metal frames adjusted weekly to maintain the correct UV-B level. For control treatments, we filtered lamps with 0.13 mm polyester film (absorbs ≤320 nm) and plants only received ambient solar UV-B. Enhanced UV-B radiation treatment lamps were filtered with 0.13 mm cellulose acetate. We changed filters weekly and monitored radiation throughout the growing season to match current ambient or ambient plus 1.6× UV-B radiation.

Foliar Sampling and Nutritional Constituents Plants were harvested before the majority of flowering and after 12 wk of treatment. Because rabbits used in this study use all parts of the plants, we harvested both leaves and stems, leaving a 3-cm stubble. We randomly selected one flat (yarrow and bluebunch wheatgrass) or one tray of containers (sagebrush) from each lamp frame (to compose five or six samples from each treatment at the midpoint of animal trials) for analyses of nutritional quality and chemical components. Samples for terpene analysis were collected between 1000 and 1130 hours to avoid diurnal variation in concentration, placed in airtight bags, frozen upon harvest to prevent breakdown of oils (−30°C), and then stored at −40°C. All other samples were lyophilized and stored in a −10°C freezer until analyses.

We evaluated samples for protein, energy, fiber, lignin, and *in vitro* dry matter digestibility (IVDMD). We determined neutral detergent fiber (NDF), acid detergent fiber

(ADF), and acid detergent lignin (ADL) of forages by using sequential detergent analysis (Goering and Van Soest 1970) with filter bags, sodium sulfite, and alpha amylase in an Ankom Fiber Analyzer (Ankom Technology, Macedon, NY, USA). Nitrogen content was determined by the Kjeldahl procedure (Bradstreet 1965), and crude protein (CP) content (%) was estimated as 6.25 times the nitrogen content (Robbins 1993). We used standard procedures for bomb calorimetry to determine gross energy content, and IVDMD was determined by using an Ankom Daisy II Incubator (Ankom Technology, Macedon, NY, USA).

Phytochemical Analyses We analyzed sagebrush and yarrow for tannin-binding capacity and terpenes. Tannin-binding capacity was determined by the bovine serum albumin (BSA) method (Robbins et al. 1987). To determine volatile terpenes, sagebrush (0.08 g) and yarrow (0.1 g) samples were extracted by using solid-phase microextraction (SPME) (Steffen and Pawliszyn 1996) with 4 ml ddH_2O and 0.65 g NaCl in a 25-ml flask. We stirred the solution at a rate of 1,100 rpm on a magnetic plate while a fused silica fiber coated with 65 um poly(dimethylsiloxane)/divinlybezene was exposed to the headspace for 35 min before injection. Splitless injection was completed over 5 min at 200°C into a Hewlett-Packard 5890II/5970 GC/MS equipped with a DB-1 60 m (0.32 mm with 0.25 μm film) column with helium as the carrier gas. Transfer line temperature and ion source was held at 250°C. We maintained column temperature at 33°C for 5 min, increased to 50°C at a rate of 2°C/min, and then to 225°C at a rate of 5°C/min. Initial identification was completed by comparing spectra with those present in the Wiley-NBS library. Further quantification and identification was accomplished by comparing sample retention times and using response factors generated from standards obtained from commercial sources (Fluka Chemicals, Milwaukee, WI, USA; Sigma-Aldrich Corporation, St. Louis, MO, USA; Indofine Chemical Company, Belle Mead, NJ, USA). Several selected compounds had mass spectra and retention data compared to the literature (Buttkus et al. 1977; Pearson 1977; Epstein and Gaudioso 1984; Hofmann et al. 1992; Gunawardena et al. 2002; Adams 2004).

Phenolic and flavonoid purification and quantification of the three forages was completed by using modified procedures of Miller (1995) that have also been utilized on pine and poplar trees (Warren et al. 2002b). We extracted lyophilized and ground plant tissue (0.5 g) with a solution of ethanol (80%) and formic acid (1%). This was then partitioned with ethyl ether to remove carotenoids and chlorophylls. The aqueous fraction was partitioned with ethyl acetate and a phenolic solution (20% ammonium sulfate, 20% ethanol, 2% metaphosphoric acid) into the acetate phase (flavonoids) and the aqueous phase (anthocyanins). We evaporated the ethyl acetate from the flavonoid fraction, and the remaining residue was dissolved in 2 ml of high-performance liquid chromatography (HPLC) grade methanol, filtered, and stored below 0°C.

We injected 10 μl of the extract onto a Dynamax UV-1 HPLC system (Varian, Inc. Walnut Creek, CA, USA) with a 5-μ C-18 column (150×4.6 mm, Varian, Inc. Walnut Creek, CA, USA) with detection at 254 nm. The mobile phase started (1.0 ml min^{-1}) at 80:10:10 (distilled water, 0.5% phosphoric acid, methanol) and was increased to 20:10:70 over 40 min. We maintained the 20:10:70 solution for 5 min. The column was cleaned with 100% methanol for 13 min and then re-equilibrated to the starting solution over 2 min. We quantified flavonoids by using response factors from standards with similar retention times on the same instrument and with the same injection method. External calibration was used, and major flavonoids compounds known to exist in plants (particularly in sagebrush and yarrow), were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and

Indofine Chemical Company (Belle Mead, NJ, USA). Compounds included esculin, chlorogenic acid, esculetin, scopoletin, coumarin, scoparone, luteolin-7-*O*-flucoside, apinegen-7-*O*-glucoside, quercetin, luteolin, 6-methyl luteolin, kaempferol, and apigenin.

For initial quantitative HPLC analysis of standards and samples, we used wavelengths 254, 280, and 320 nm. Final analysis was completed at 254 nm, which has been shown to be the UV-B wavelength useful in analyzing a wide range of phenolics in methanol or acidified methanol (Macheix et al. 1990; Waterman and Mole 1994).

Preference and Digestibility Feeding Trials Pygmy rabbits used for nutrition studies were bred in captivity at Washington State University from animals originally captured in Idaho. Six adult animals (two female, four male) ranging in age from 1 to 2 yr and in weight from 350 to 464 g were available from which 2–4 were used for each trial. Two female and two male eastern cottontails were captured on the grounds at E. H. Steffen Center. All were at least 1-yr old at capture and they ranged in weight from 950 to 1,283 g. When not used in experiments, animals were housed in stainless steel rabbit cages and fed an *ad libitum* (ad lib) maintenance diet, a mix of fresh forbs, tap water, and supplied with a mineral block. All experimental protocol and husbandry practices were approved by the WSU Institutional Animal Use and Care Committee (#3291).

We conducted preference trials to determine whether animals could detect and make decisions based on chemical changes induced by enhanced UV-B radiation. By using standard procedures for cafeteria-style preference tests, we offered foliage produced under ambient and enhanced UV-B regimes (Molloy and Hart 2002). Animals were gradually shifted from their maintenance diet to each experimental diet over 1–2 wk, and during the last 2 d of pretrial, we moved animals into metabolic crates. During trials, we offered equal amounts (dry mass basis) of tissue grown under ambient and enhanced levels of UV-B radiation for each day of 5 d per plant species to 2–3 adult pygmy rabbits and 3–4 adult cottontail rabbits. We offered fresh plant material 2–6 times per day to maintain freshness and to limit the release of volatiles from plants. Orts were dried and weighed to determine dry mass consumed. The software Prefer for Windows based on Johnson's (1980) method with usage and availability, was used to evaluate preference of plants treated with ambient vs. supplemental UV-B grown plants.

Separate total-collection digestion trials were used to determine if chemical changes enhanced by UV-B radiation had physiological effects on the two rabbit species. We gradually shifted animals from their maintenance diet to each experimental diet over 1 to 2 wk and isolated animals in metabolic crates 2 d before the start of trial. For each day of a 4- or 5-d digestion trial, we offered 2–4 adult pygmy rabbits and 3–4 adult cottontail rabbits fresh forage 2–6 times per day. Forages were corrected for dry matter each day, and we collected all urine, feces, and orts, and determined energy balance. During the sagebrush digestion trials, we supplemented both species of rabbits with dry pellets because the cottontails would not eat any sagebrush alone. Animals were offered 5% body weight of pellets, and animals consumed all pellets offered. Using standard procedures (Goering and Van Soest 1970; Robbins 1993), we calculated dry matter, fiber, protein, and apparent energy digestibility, and we compared between plant treatments for each species (ambient vs. enhanced UV-B irradiated plants) and animal species.

Statistical Analyses One-way analysis of variance (PROC GLM, SAS Institute, Inc., 2005) was performed to test for significant treatment effects on plant components as well as the response of animals. We tested for differences between treatments for all nutritional components and for each individual terpene and phenolic. Differences between treatment

Table 1 Mean and standard deviation of neutral detergent fiber (NDF, %), acid detergent fiber (ADF, %), acid detergent lignin (ADL, %), crude protein (CP, %11), energy (kJ/g), tannin-binding capacity (BSA, mg BSA ppt/mg), and *in vitro* dry matter digestibility (IVDMD, %) of sagebrush (*Artemisia tridentate*), yarrow (*Achillea millefolium*), and bluebunch wheatgrass (*Pseudoreogneria spicata*) irradiated with 1× and 1.6× UV-B radiation

Species	*N*	Radiation	NDF	ADF	ADL	CP	Energy	IVDMD	BSA
Big sagebrush	6	1× UV-B	38.82±2.24	18.72±0.94	13.36±0.53*	17.99±0.97	5,195±55.36	71.46±2.67*	0±0
	6	1.6× UV-B	37.11±1.55	18.11±0.81	10.49±2.54	18.79±0.49	5,181±94.80	66.78±3.10	0±0
Yarrow	6	1× UV-B	33.71±1.34	17.15±0.77	3.48±0.57	19.76±1.34	3,935±269.27	73.73±2.72	0±0
	6	1.6× UV-B	34.19±3.02	16.67±1.43	3.66±0.37	19.21±0.76	4,025±184.58	74.24±3.95	0±0
Bluebunch wheatgrass	5	1× UV-B	37.85±4.67	15.09±0.64	3.14±0.25	29.57±1.79	4,390±94.09	67.63±4.65	–
	5	1.6× UV-B	34.73±0.98	15.70±0.70	3.01±0.51	28.63±1.14	4,506±113.68	69.13±1.24	–

An asterisk denotes a significant difference between radiation levels for that plant species (P=0.05).

and animal species were also tested by using treatment*species as the interaction. All percent data were arcsin-transformed before analyses.

Results

Forage Quality In general, the nutritional quality of forages was not affected by supplemental UV-B radiation. Neither yarrow or bluebunch wheatgrass showed any changes in neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), crude protein (CP), energy, or *in vitro* dry matter digestibility (IVDMD) ($P>0.05$, Table 1). Only sagebrush showed changes with higher UV-B radiation, which caused lignin ($P=0.021$) and IVDMD ($P=0.018$, Table 1) to decrease. Sagebrush and yarrow showed no precipitate in the BSA assay, indicating no biological response to possible tannins in those plants.

Phytochemistry We identified 18 different volatile compounds from sagebrush and yarrow (Table 2). There was no significant change correlated with increased UV-B radiation for the major terpenes in either plant (Fig. 1) with the exception of E-β-santolina epoxide, which decreased ($P=0.002$) from 450 ng/ml to 55 ng/ml in yarrow treated with enhanced (1.6×) UV-B.

Table 2 Volatile terpenes found in yarrow (*Achillea millefolium*) and sagebrush (*Artemisia tridentata*), retention time (min), retention indices (RI) calculated as kovats retention index using a series of straight chain alkanes and identification (ID) made by, R: comparison of mass spectra and retention indices of authentic substances or, L: by comparison of mass spectra and retention data from literature

RT	Compound Name	Amount (μg/g)		RI	ID
		Yarrow	Sagebrush		
20.80	Santolina triene		0.23	895	L
21.70	α-pinene	0.75		915	R,L
22.71	Camphene		4.55	932	R
23.78	Sabinene	1.07		957	R
23.86	Arthole		17.40	959	L
24.80	Unknown	1.45	6.05	981	R
25.20	α-phelladrene		2.04	985	R
25.80	α-terpinene	0.85		997	R
26.20	1,8-cineole	4.13	7.14	1,006	R
27.03	E-β-cantolina epoxide	0.45	15.90	1,032	L
27.25	Z-β-santolina epoxide	0.45	11.68	1,037	L
27.37	γ-terpinene	0.68		1,045	R
28.80	α-thujone	3.90		1,074	R
29.20	β-thujone	0.50		1,082	R
30.19	Camphor	6.71	20.25	1,106	R
30.38	E-β-methyl santolinate		32.80	1,114	L
30.91	Z-β-methyl santolinate		26.05	1,131	L
31.41	Artemisyl acetate	15.94	33.92	1,146	L

Literature (L) used for identification (ID) include: Buttkus et al. 1977; Pearson 1977; Epstein and Gaudioso 1984; Hofmann et al. 1992; Gunawardena et al. 2002; Adams 2004.

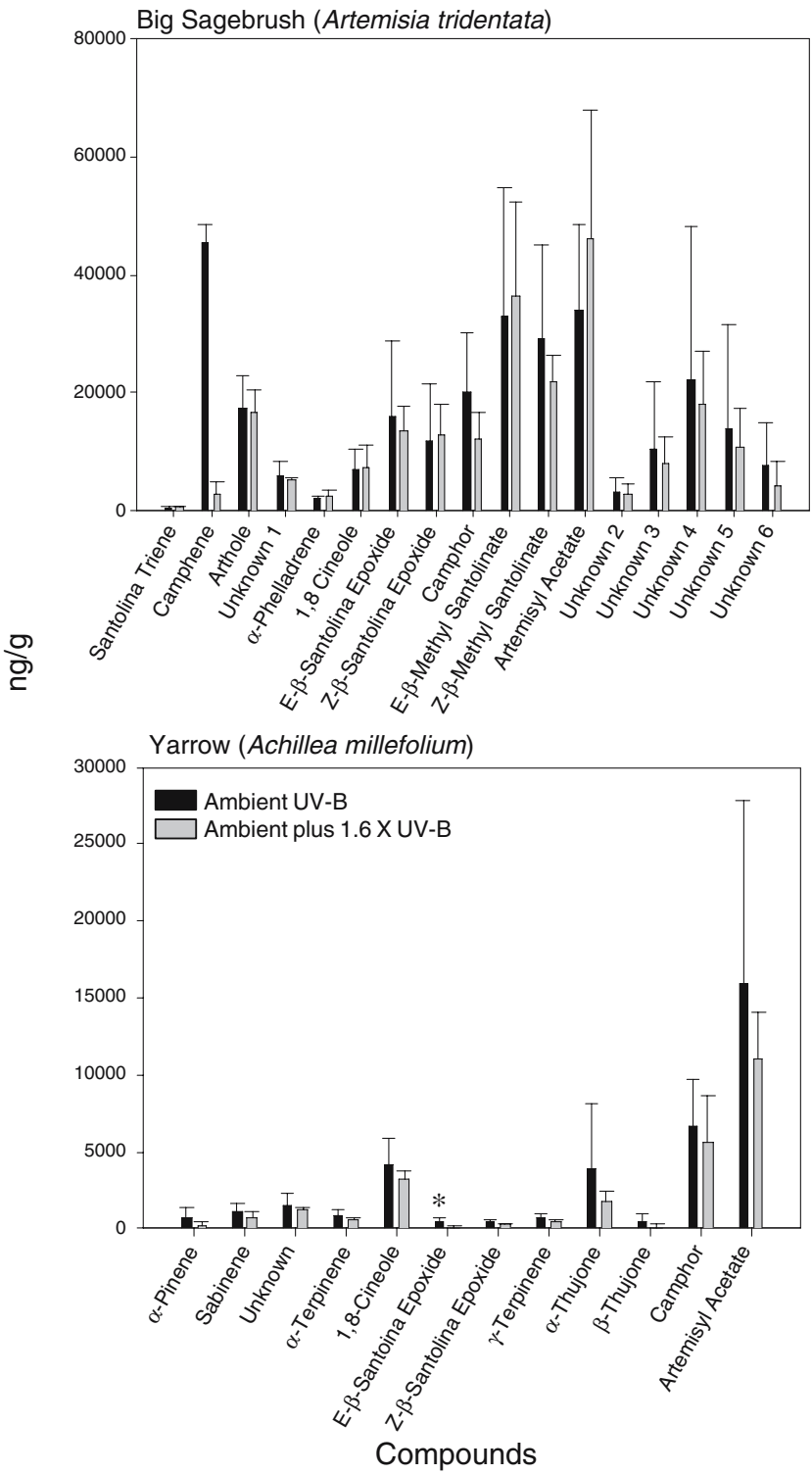

Big Sagebrush (Artemisia tridentata)
ng/g
80000
60000
40000
20000
0
Santolina Triene
Camphene
Arthole
Unknown 1
α-Phelladrene
1,8 Cineole
E-β-Santolina Epoxide
Z-β-Santolina Epoxide
Camphor
E-β-Methyl Santolinate
Z-β-Methyl Santolinate
Artemisyl Acetate
Unknown 2
Unknown 3
Unknown 4
Unknown 5
Unknown 6
Yarrow (Achillea millefolium)
Ambient UV-B
Ambient plus 1.6 X UV-B
30000
25000
20000
15000
10000
5000
0
*
α-Pinene
Sabinene
Unknown
α-Terpinene
1,8-Cineole
E-β-Santoina Epoxide
Z-β-Santolina Epoxide
γ-Terpinene
α-Thujone
β-Thujone
Camphor
Artemisyl Acetate
Compounds

Fig. 1 Mean±SD (vertical bars) of volatile terpenes in ng/g of big sagebrush (N=6) and yarrow (N=6) irradiated with ambient or ambient plus 1.6× supplemental UV-B radiation for Pullman, WA, USA. An asterisk indicates a significant treatment effect ($P \leq 0.05$)

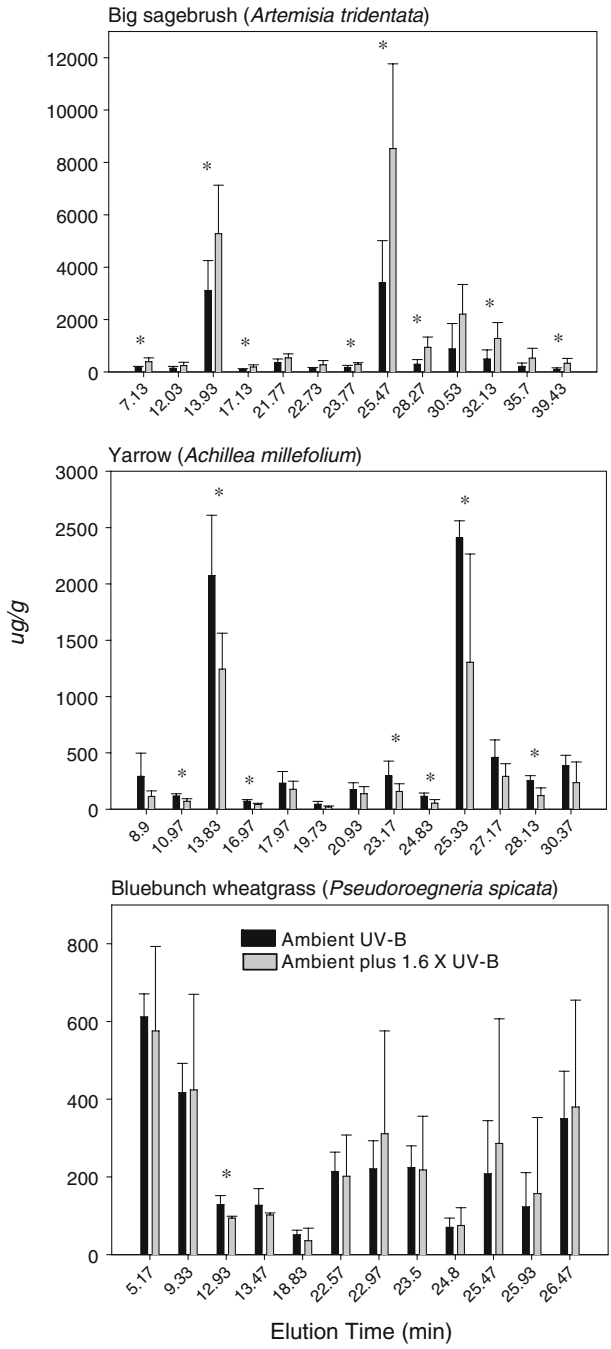


Fig. 2 Mean±SD (vertical bars) of flavonoid compounds in ug/g of big sagebrush (N=6), yarrow (N=6), and bluebunch wheatgrass (N=5) irradiated with ambient or ambient plus 1.6× supplemental UV-B radiation for Pullman, WA, USA. An asterisk indicates significant treatment effects ($P \leq 0.05$)

Table 3 Mean and standard deviation of intake (g/d), dry matter, fiber, protein, and apparent digestible energy (%) of sagebrush (*Artemisia tridentata*), yarrow (*Achillea millefolium*), and bluebunch wheatgrass (*Pseudoreogneria spicata)* irradiated with 1× and 1.6× UV-b radiation and fed to pygmy rabbits (*Brachylagus idahoensis*) and eastern cottontails (*Sylvilagus floridanus*)

Plant	Animal	*N*	Radiation	Intake	Dry Matter Digestibility	Fiber Digestibility	Protein Digestibility	Apparent Digestible Energy
Big sagebrush	Pygmy rabbit	4	1× UV-B	15.5±10.5*	60.42±5.9	32.43±12.6	67.84±7.5	63.17±5.5
		3	1.6× UV-B	21.0±12.4	60.38±8.9	30.35±18.4	69.08±3.4	65.90±8.2
	Eastern cottontail	3	1× UV-B	5.4±1.6	62.32±20.1	38.73±32.46	75.73±10.6	62.85±28.3
		3	1.6× UV-B	4.4±0.5	70.17±13.5	51.95±21.8	76.82±13.2	76.67±9.3
Yarrow	Pygmy rabbit	2	1× UV-B	29.9±5.2	48.19±6.8*	22.93±7.3	48.64±6.8*	54.98±2.3
		2	1.6× UV-B	31.0±6.0	50.12±12.4	23.31±17.3	49.62±18.2	54.21±11.9
	Eastern cottontail	2	1× UV-B	28.4±9.4	65.72±21.7	38.16±38.6	80.36±13.1	65.40±25.3
		2	1.6× UV-B	25.6±8.1	73.74±5.0	55.35±15.9	83.12±1.8	76.23±4.5
Bluebunch wheatgrass	Pygmy rabbit	2	1× UV-B	23.2±7.3*	59.76±3.7*	29.86±7.4*	75.78±1.0	58.81±1.8*
		4	1.6× UV-B	22.4±5.1	62.97±4.2	24.61±8.0	77.85±2.8	64.23±3.5
	Eastern cottontail	2	1× UV-B	32.8±9.6	71.44±6.3	51.43±11.6	79.21±8.0	72.84±5.9
		2	1.6× UV-B	41.1±6.6	68.21±0.6	40.23±0.8	78.78±2.2	69.02±3.1

An asterisk denotes a significant difference in digestibility between rabbit species for that plant species (P=0.05)

Although found in lower concentration than in the dicots sampled, bluebunch wheatgrass contained a large number of phenolic compounds (Fig. 2), but only one was influenced by enhanced UV-B radiation (P=0.048). Sagebrush and yarrow phenolics, however, did respond to increased UV-B radiation. In sagebrush, eight of the 13 predominant phenolics increased with higher UV-B radiation (all P<0.035, Fig. 2). In contrast, seven of the 14 predominant phenolics decreased in yarrow (all P<0.046, Fig. 2).

Animal Response When simultaneously offered plants from each treatment, neither pygmy rabbits nor cottontails showed a preference for 1× or 1.6× UV-B irradiated plants of sagebrush (pygmy rabbits: F=1.00, P=0.42; cottontails: F=0.25, P=0.67), yarrow (pygmy rabbits: F=1.00, P=0.36; cottontails: F=1.00, P=0.39), or bluebunch wheatgrass (pygmy rabbits: F=1.00, P=0.42; cottontails: F=1.00, P=0.42). Similarly, in digestion trials where treatments were fed separately, rabbits also ate the same amount of treated and untreated plants (all P's>0.05, Table 3). After consuming plants, both rabbits also digested ambient and supplemental UV-B irradiated plants equally for each plant species (all P>0.05, Table 3). However, regardless of treatment, the larger cottontail rabbit was better able to digest yarrow and bluebunch wheatgrass than the pygmy rabbit. The cottontails achieved a higher dry matter (P=0.037) and protein (P=0.003) digestibility than pygmy rabbits fed yarrow (Table 3). During bluebunch wheatgrass trials, cottontails also achieved a higher dry matter digestibility (P=0.018), fiber digestibility (P=0.010), and apparent digestible energy (P=0.011, Table 3).

Discussion

UV-B radiation, artificially enhanced to the levels predicted for temperate North America representing a 15% ozone depletion, influenced flavonoid concentrations of a forb and shrub common to rangelands of North America, but had minimal effects on other aspects of nutritional chemistry. In turn, neither a specialist nor generalist lagomorph consuming these plants showed preference or digestive response to the UV-B treatments.

Phytochemistry In our study, as in previous ones (Tegelberg et al. 2002; Warren et al. 2002b; Ambasht and Agrawal 2003), the accumulation of flavonoids was the most common response to UV-B radiation. In sagebrush, eight of 13 flavonoids detected increased significantly with higher UV-B levels. Increases in flavonoids with UV-B radiation are expected, because they are known to protect plants by attenuating UV-B radiation in the leaf epidermis, thus reducing internal leaf damage (Brandt et al. 1995). Some flavonoids, such as triterpenoids, flavonoid-aglycones, kaempferol-2-0-β-glucuronic acid, and quercetin-3-0-β-glucuronic acid, can deter feeding by mammals (Halaweish et al. 2003; Laitinen et al. 2004), and others can increase fat excretion and reduce *in vitro* fermentation (Bravo et al. 1994). However, flavonoids are a diverse group of 4,500 compounds, and the effects of UV-B radiation on them may not be universal in plants. In our yarrow, seven of 13 flavonoids decreased, and in bluebunch wheatgrass, only one of 12 flavonoids decreased.

Condensed tannins comprise another subclass of phenolics. Condensed tannins are bitter, function as feeding deterrents, and can bind to proteins in plants, making some protein and energy functionally unavailable to herbivores that feed on them (Robbins et al. 1987). Higher UV-B radiation may compound the biological effects of tannins as plants growing in the shade of coniferous trees have shown lower levels of tannins than those growing in full

sun (Hartly et al. 1997). However, we detected no tannin-binding capacity of phenolics in either sagebrush or yarrow.

Terpenes are known to inhibit or destroy gut flora (Burritt et al. 2000), act as a feeding deterrent (Sinclair et al. 1988; Gershenzon and Croteau 1991), cause acidosis (Cork and Foley 1991), or act as a cue for animals to avoid more toxic secondary compounds (Bray et al. 1991; Lawler et al. 1999). Previous studies indicated that when UV-B radiation was filtered out, terpene concentration was altered in *Grindelia chiloensis* (Zavala and Ravetta 2002) and leaves of *Ocimum basilicum* (Johnson et al. 1999). However, in our experiments, enhanced UV-B radiation did not affect overall terpene composition and quantity in sagebrush or yarrow. Similarly, Tegelberg et al. (2002) found that enhanced UV-B radiation did not influence terpenes in *Betula pendula.* Plants may be less responsive to increases when exposed to a relatively high ambient level of UV-B radiation because target compounds and mechanisms may already be saturated at ambient levels of UV-B radiation (Day 2001).

Like flavonoids, lignin forms via the shikimate acid pathway, and gives structural support to plants. Lignin is insoluble and nondigestible by mammals and thus limits their ability to digest the plant cell wall (Van Soest 1994). Although little-studied, lignin in grasses and trees (Rozema et al. 1997; Cybulski et al. 2000) has been shown to increase with UV-B radiation. We expected plants that we irradiated with supplemental UV-B radiation to have more lignin, especially the grass that is not presumably defended by secondary compounds but rather by a thick cell wall. However, enhanced UV-B radiation did not alter any component of the cell wall (NDF, ADF, or lignin), except in sagebrush, where lignin declined by 21%. We harvested all plant tissue as new growth <120 days. At harvest, the stems of sagebrush were still green and nonwoody, and bluebunch wheatgrass was a lush green with only 3% lignin, which was lower than lignin content of bluebunch wheatgrass collected in the field during the spring (4–6%, Thines et al. 2004). Thus, any differences in lignin caused by UV-B in bluebunch wheatgrass might be only detectable at maturity.

When plants allocate resources to producing secondary plant metabolites, protein is sacrificed. A negative relation has been found between phenolics and nitrogen (Price et al. 1989), and protein and nitrogen decrease in various grasses, forbs, and tree species with enhanced UV-B radiation (Zavala and Ravetta 2002; Milchunas et al. 2004). However, although enhanced UV-B radiation altered flavonoids of sagebrush and yarrow, it did not alter protein content of any of the plants in our study.

Animal Response Although terpenes, phenolics, cellulose, and lignin can either be toxic or reduce the nutrients gained from forages by mammalian herbivores, the changes in these in plants exposed to enhanced UV-B radiation in our study were too small and inconsistent to influence the behavior and digestion of pygmy rabbits and eastern cottontails. In fact, the variation in plant chemistry caused by two levels of UV-B radiation was within the range of variation commonly experienced among plants, plant parts, and in different seasons by mammalian herbivores. For example, in a field study assessing pygmy rabbit forages, fiber content ranged from 39% in big sagebrush to 70% in needlegrass (*Stipa* spp.), and protein ranged from 5.4% in bluebunch wheatgrass to 10.9% in big sagebrush (Thines et al. 2004). Likewise, Thines et al. (2004) found that nutritional components differed significantly among seasons for four common pygmy rabbit forages including sagebrush and bluebunch wheatgrass. In fact, the differences found in nutritional components among seasons was greater than differences we detected in our measurements of plants grown under different amounts of UV-B. These data perhaps suggest that alterations in plant chemistry and

nutritional components caused by increasing UV-B levels are not likely to be greater than variations already found in nature.

If our rabbits did detect differences in plant chemistry caused by enhanced UV-B radiation, they may have been able to mitigate chemical effects by selecting plant tissue on a finer scale than measured in our experiment, i.e., subtly avoiding plants and plant parts with higher content of plant secondary metabolites and fiber. For example, other lagomorphs and rodents select plant parts that have higher protein and energy but lower concentrations of terpenes and phenolics (Gershenzon and Croteau 1991; Zhang and States 1991; Palo et al. 1992).

Additionally, mammalian herbivores, such as rabbits, that house microorganisms in their digestive tract, may not respond to changes in plant chemistry as dramatically as do monogastric mammals or insects, because microorganisms may be able to detoxify or ameliorate effects of plant chemistry and can adapt to changes in plant flora (Launchbaugh 1996). For example, monogastric rats demonstrated aversive behavior to kaempferol-2-0-β-glucuronic acid and quercetin-3-0-β-glucuronic acid extracted from leafy spurge (*Euphorbia esula*), whereas foregut-fermenting cattle did not (Halaweish et al. 2003). Although animals often need time to develop conditioned aversions to toxic chemicals through postingestive feedback (Provenza et al. 1992; Launchbaugh 1996), our pretrial period was designed to give the rabbits adequate time to detect and respond to increased levels of toxic plant chemicals.

In summary, responses of plant chemistry to UV-B radiation were neutral to mixed. Although flavonoids were consistently higher in sagebrush and lower in yarrow, such changes did not influence protein-precipitating capacity or *in vitro* dry matter digestibility. Thus, artificial application of UV-B radiation, representing a 15% ozone depletion (1.6×), did not alter the secondary compounds and nutritional components of sagebrush, yarrow, and bluebunch wheatgrass enough to change the behavior or digestion of a generalist and a specialist lagomorph.

Acknowledgments The authors thank Gary Radamaker for assistance with field work and maintenance of greenhouse plants. This project was funded with support of the United States Department of Agriculture's Ultraviolet-B Monitoring and Research Program at Colorado State University.

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J Chem Ecol (2007) 33:1041–1048
DOI 10.1007/s10886-007-9285-2

Inhibition of Proteinase Inhibitor Transcripts by *Leptinotarsa decemlineata* Regurgitant in *Solanum lycopersicum*

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Received: 28 November 2006 / Revised: 21 February 2007 / Accepted: 14 March 2007 /
Published online: 3 April 2007
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Abstract One mechanism by which plants defend themselves against insect herbivores is the production of plant proteinase inhibitors, which can inhibit digestion in the midgut, thus affecting growth and survival. In this work, the effect of Colorado potato beetle (CPB) [*Leptinotarsa decemlineata* (Say)] regurgitant on *Solanum lycopersicum* defenses was investigated. When regurgitant from fourth-instar CPB was applied to wounded *S. lycopersicum* leaves, the wound-induced transcripts for the proteinase inhibitors *pin1* and *pin2* were reduced. Boiling the regurgitant abolished its ability to reduce the *pin* transcripts. Ultrafiltration of the regurgitant demonstrated that it contained a component between 10 and 30 kDa molecular weight that inhibited wound-induced *pin1* and *pin2* expression, suggesting that it may be a protein. This may represent a mechanism that the CPB has evolved to elude the plant's induced response to infestation.

Keywords Herbivore · Colorado potato beetle · Regurgitant · Proteinase inhibitor

Introduction

The Colorado potato beetle [CPB; *Leptinotarsa decemlineata* (Say), Coleoptera: Chrysomelidae] is a serious pest of solanaceous crops in the USA. In *Solanum tuberosum* (potato), volatiles produced in the plant upon attack by CPB result in the attraction of the CPB predator *Perillus bioculatus* (F.) (Heteroptera: Pentatomidae) (Weissbecker et al. 1999). This response appears to be a part of the insect/plant interaction because mechanical wounding alone does not produce the same set of volatiles or attraction of the predator.

Elicitors isolated from the regurgitant of pest insects applied to mechanically wounded leaves often result in a plant response that mimics the response to insect feeding. For example, a β-glucosidase was isolated from *Pieris brassicae* (L.) regurgitant (Mattiacci et al.

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1995). Volicitin (Albourn et al. 1997), a fatty acid–amino acid conjugate (FAC), is synthesized by the insect from a plant fatty acid and an amino acid from the insect (Pare et al. 1998). When placed on a wounded leaf, this component of *Spodoptera exigua* (Huber) saliva elicited volatiles similar to those produced by insect infestation rather than by mechanical injury. In addition, glucose oxidase (GO) from the salivary glands of *Helicoverpa zea* (Boddie) (Eichenseer et al. 1999; Musser et al. 2002) and additional FACs from other lepidopteran species (Pohnert et al. 1999; Halitschke et al. 2001) all either enhance or reduce defense responses when applied to a wounded leaf. Thus, it appears that a number of insect-produced secretions may affect plant response to insect attack.

Baldwin's lab (Halitschke et al. 2001; Schittko et al. 2001) has examined the effect of regurgitant from *Manduca sexta* (L.) on *Nicotiana attenuata*. They have isolated FACs that mimic the effect of the regurgitant (Halitschke et al. 2001). One gene coding for threonine deaminase, normally induced by wounding, was inhibited by regurgitant (Schittko et al. 2001). Other genes were amplified by regurgitant, whereas others inhibited by wounding were reduced further. Generally, nicotine expression was inhibited by GO (Musser et al. 2002), whereas FACs induced plant defense genes.

Little is known about the components of CPB regurgitant. Although regurgitant enhances peroxidase and polyphenol oxidase activity, ethylene is also increased in regurgitant-treated wounded leaves of potato (Kruzmane et al. 2002). We have tested the effects of CPB regurgitant on wound-induced genes in *Solanum lycopersicum* and have found that the wound-induced proteinase inhibitor transcripts *pin1* and *pin2* are inhibited by CPB regurgitant. In addition, the inhibitory component in the regurgitant appears to be between 10 and 30 kDa molecular weight (MW).

Methods and Materials

Plant Material Solanum lycopersicum var. Rutgers were germinated from seed and transplanted to individual 4-in pots containing Metromix® (Scotts, Marysville, OH, USA) and osmocote® (Scotts). Plants were grown for approximately 4 weeks in a greenhouse until they had at least eight leaves.

Regurgitant Colorado potato beetle were reared on *S. lycopersicum*. Regurgitant was collected from the oral cavity of fourth-instar CPB with a P200 Pipetteman (Gilson, Middleton, WI, USA). The regurgitant was centrifuged for 5 min at 10,000×*g*, and the supernatant was collected and stored at −80°C.

Wounding, Regurgitant Treatment, and Infestation Mechanical wounding involved crushing a leaf with pliers, while avoiding the major veins, at 1-cm increments. Regurgitant was diluted in water or phosphate-buffered saline at the concentration specified in the figure legends. One hundred microliters of diluted regurgitant per leaf was added within minutes to the wounds, and the plants were incubated for 4 hr unless otherwise noted. To test the activity after boiling, the regurgitant was resuspended at a 1:3 dilution with water and heat inactivated for 5 min at 100°C in a heat block. The boiled regurgitant was centrifuged for 1 min, and the supernatant was collected and applied to a wounded leaf. Regurgitant was centrifuged at 20,000×*g* for 20 min at 8°C, and 250 μl of the supernatant was subjected to ultrafiltration by using Microcon™ centrifugal filtration devices (Millipore, Bedford, MA, USA) with 3, 10, 30, and 100 kDa MW cut-off (MWCO) regenerated cellulose membranes. Samples were centrifuged at 7,000×*g* until sufficient material passed through the membrane

for assay. The filtrate was collected and frozen at −20°C until diluted and applied to wounded leaves.

Five hundred microliters of regurgitant was diluted with 2 ml of water and loaded onto a Sep-Pak Light™ C18 solid phase extraction cartridge (Waters, Milford, MA, USA) equilibrated with 5% acetonitrile (MeCN) in water. After the sample was loaded, the cartridge was washed with 3 ml of 5% MeCN and eluted stepwise with 3 ml each of 40, 80, and 100% MeCN. Each fraction was collected in a polypropylene tube, dried by vacuum centrifugation, dissolved in a small amount of ethanol, and brought up to 500 μl with water prior to assay. To determine the time course of CPB-infested plants, the fifth leaf from the bottom was covered with a fine mesh sleeve and ten third- to fourth-instar CPB larvae that had been starved for 4 hr were added. The insects were allowed to feed for 45 min, at which time they were removed and the plants were held for 0, 1, 2, 4, 7, and 24 hr. Approximately 10% of the leaf area had been eaten after 45 min of infestation.

RNA Isolation and Northern Blots RNA was isolated from treated leaves by rapidly freezing in liquid nitrogen using the methodology of Chang et al. (1993). Plant tissue was ground with a mortar and pestle and extracted with vigorous shaking in a buffer preheated to 65°C containing 2% cetyl hexadecyltrimethylammonium bromide, 2% polyvinylpyrrolidone, 100 mM Tris pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/l spermidine, and 2% β-mercaptoethanol. This mixture was chloroform extracted ×2. The aqueous layer was precipitated overnight at 0°C with 1/4 vol of 10 M LiCl. The pellet was collected by centrifugation at 11,950×*g* for 20 min and resuspended in 1.0 M NaCl, 0.5% sodium dodecyl sulfate, 10 mM Tris pH 8.0 and 1 mM EDTA. The RNA was extracted once with chloroform and precipitated in 2 vol of 95% ethanol.

RNA was run on formaldehyde denaturing agarose gels and transferred onto Hybond N+ (Amersham, Buckinghamshire, England) by using capillary transfer as described in Sambrook et al. (1989). *pin1* and *pin2* were acquired from the Boyce Thompson Institute Tomato EST Distribution Center (Ithaca, NY, USA) as LET41L12 and LET21I5, respectively. These cDNAs were cloned into pBluescript, and were amplified by PCR using the T7 and T3 promoter primers. ^{32}P-labeled *pin1* and *pin2* transcript was produced with an *in vitro* transcription kit from Ambion (Austin, TX, USA), and the unincorporated ^{32}P was removed by using NucAway spin columns (Ambion). Northern hybridization was performed with Expresshyb hybridization solution (Clontech, Palo Alto, CA, USA) and washed as described in the instruction manual. The ribosomal RNA (rRNA) probe came from J. Davis (University of Florida, Gainesville). It was cloned into pGEM-T, prepared as described for the other probes, and used to equalize loading of the lanes. Relative expression of *pin1* and *pin2* was calculated by quantifying volume in the band using Imagequant (Molecular Dynamics, Sunnyvale, CA, USA) and dividing by volume of the rRNA band. Each RNA sample contained material from two plants. For Figs. 2, 3 and 4, the experiments were duplicated, although the results of a single experiment are presented.

Results

Mechanical wounding induced *pin1* and *pin2* (Fig. 1). The samples from different experiments were divided by their control, so the relative expression represents the fold increase in expression. The addition of CPB regurgitant to the wounded leaf resulted in a reduction of the amount of wound-induced transcript present. This is particularly apparent

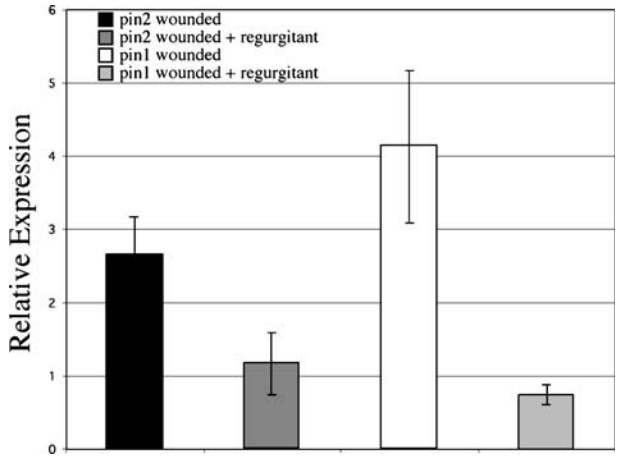


Fig. 1 Wound-induced *pin1* and *pin2* were inhibited by the addition of regurgitant. These data represent five separate experiments. The fifth or sixth leaf was wounded and regurgitant was added. Plants were incubated for 4 hr, at which time the wounded leaf was harvested and frozen in N_2. RNA was isolated and Northern blots were performed. To calculate relative expression between experiments, the wounded sample or the wounded plus regurgitant sample was divided by the control from the appropriate experiment. *Error bars* represent standard error. $N=5$ for wounded and $N=10$ for wounded plus regurgitant treatment

for *pin1*. Because the equal variance test failed for the *pin2* data, the nonparametric Mann–Whitney rank sum test was performed on the raw data with SigmaStat 2.0. This test showed that the addition of regurgitant to mechanically wounded leaves significantly lowered transcript levels for both *pin1* ($P=0.004$) and *pin2* ($P=0.028$) when compared to mechanical wounding alone. Boiling of the regurgitant before application to the wounded leaf inactivated the inhibitory factor (data not shown).

A time course was performed to determine whether the regurgitant is slowing the production of the transcript with time. Fig. 2 shows that the amount of *pin1* and *pin2* that is produced is greatest after 24 hr. Production of *pin1* and *pin2* is appreciably reduced at 8 and 24 hr by the addition of regurgitant.

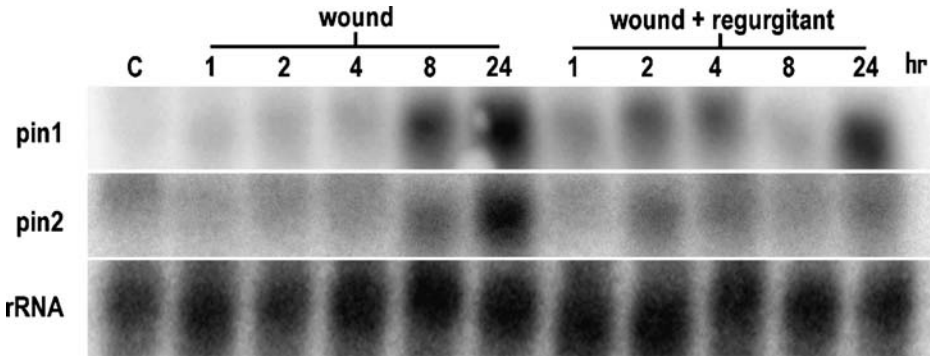


Fig. 2 Wound-induced *pin1* and *pin2* were inhibited by the addition of regurgitant at 8 and 24 hr. The sixth leaf from the bottom was wounded and 100 μl of regurgitant diluted in water 1:3 was or was not added. The plants were incubated for 0, 1, 2, 4, 8, or 24 hr. The wounded leaves were harvested and frozen in N_2. RNA was isolated and Northern blots were performed

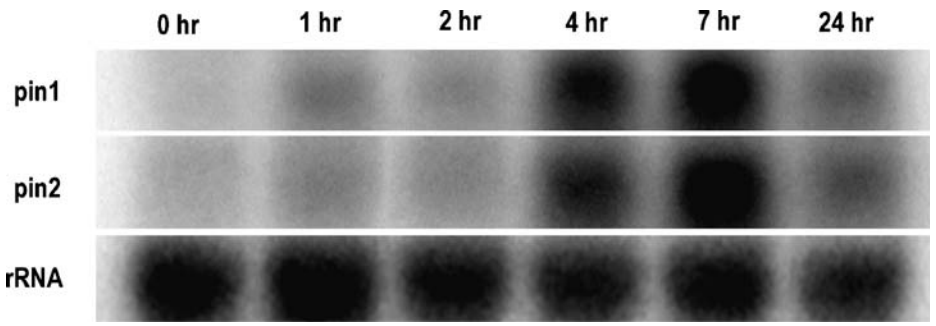


Fig. 3 Infestation of *S. lycopersicum* leaves with CPB resulted in the expression of *pin1* and *pin2* at 4 and 7 hr. The fifth leaf from the bottom was infested with ten third- to fourth-instar CPB for 45 min and held for an additional 0, 1, 2, 4, 7, or 24 hr. The wounded leaves were harvested and frozen in N_2. RNA was isolated and Northern blots were performed

Is the negative effect of the regurgitant apparent in leaves that have been infested by CPB? A time course was performed by using plants that had been infested for 45 min, after which approximately 10% of the leaf area had been eaten. The amount of leaf injured is less than the amount affected by our wounding regime, so it is difficult to compare wounding and infestation. Fig. 3 demonstrates that transcripts were indeed produced in leaves that have been infested with CPB. The amount of transcript for *pin1* and *pin2* peaked at 7 hr.

Finally, two different approaches were made to further characterize the component of the regurgitant responsible for the inhibition of *pin1* and *pin2* transcript. First, regurgitant was loaded onto a C18 solid phase extraction cartridge, and bound material was eluted with increasing concentrations of acetonitrile. No inhibitory activity was recovered using this approach. However, we also subjected regurgitant to ultrafiltration. The results are shown in Fig. 4. The inhibitory activity passed freely through a 30-kDa MWCO membrane, but not through 3- or 10-kD MWCO membranes.

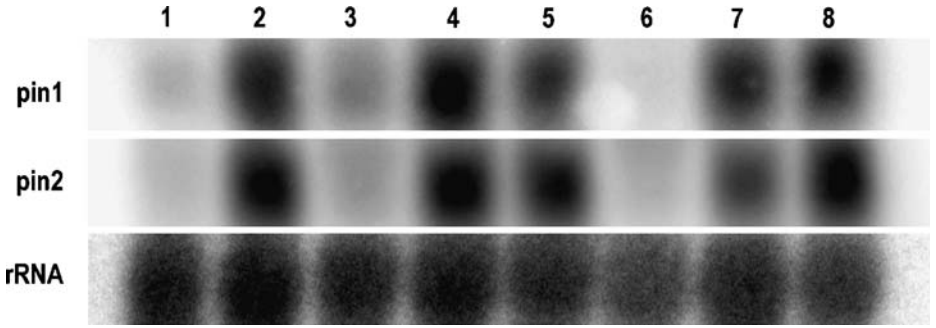


Fig. 4 A component of CPB regurgitant between 10 and 30 kDa MW results in the inhibition of *pin1* and *pin2*. The ninth leaf from the bottom was wounded and regurgitant diluted 1:2 in phosphate-buffered saline (PBS) was or was not added. *Lane 1*, unwounded; *2*, wounded + PBS; *3*, wounded + regurgitant diluted in PBS 1:2; *4*, wounded + filtrate from 3 kDa; *5*, wounded + filtrate from 10 kDa; *6*, wounded + filtrate from 30 kDa; *7*, wounded + filtrate from 100 kDa; and *8*, regurgitant remaining on top of 100-kDa filter. The plants were incubated for 4 hr and the wounded leaf was harvested. RNA was isolated and Northern blots were performed

Discussion

Addition of CPB regurgitant to wounded leaves results in the inhibition of *pin1* and *pin2* wound-induced transcripts. However, although infestation by CPB results in the expression of *pin1* and *pin2*, the addition of regurgitant does not fully mimic the insect/plant interaction. It is difficult to wound tissue in the same manner as insect feeding because the insect feeds at a comparably slower rate and the resulting wounds are different. The Mecworm, a mechanical device developed by Mithofer et al. (2005), mimics the wounding produced by a caterpillar and consequently results in a qualitatively similar volatile mix as produced by the insect. Because that work was done by replicating a single insect species on lima bean, it is not clear how general this result may be. It does suggest that more natural wounding is necessary to tease out the mechanical reaction to insect feeding from any insect-produced compounds. Alternatively, other compounds that allow production of *pin1* and *pin2* may not survive the isolation and storage of the regurgitant, in which case the regurgitant may contain a complex mix of plant reactive components. Finally, CPB were starved for 4 hr before they were used for infestation. This is necessary to entice the insects to feed quickly on the plant, but it may affect the contents of the CPB digestive system, altering components of the regurgitant and affecting the inhibition of *pin1* and *pin2*.

The size of the inhibitory compound appears to be between 10 and 30 kDa MW; given the size and also that it is inactivated by boiling suggests that it is a protein. One perplexing question is why the inhibitory factor was not present in the 100-kDa MW filtrate? We speculate that the factor is somewhat labile and that perhaps a protease copurifies with the factor in the 100-kDa MW filtrate but that the factor is protected by other proteins in the unfractionated sample.

The enzyme GO is present in salivary extracts from the caterpillar of *H. zea*, and this enzyme inhibits the defense compound nicotine in tobacco (Musser et al. 2002). However, the MW of GO is greater than 60 kDa (Eichenseer et al. 1999), so perhaps the component of CPB is a different protein. Interestingly, salivary gland extract from *H. zea* moderately inhibits trypsin inhibitor in *S. lycopersicum*, whereas GO does not (Musser et al. 2005). Perhaps additional components are present in *H. zea* that have not been identified.

Colorado potato beetle larval midgut contains cysteine and aspartate proteinases (Wolfson and Murdock 1987). However, a serine proteinase has also been detected in CPB (Novillo et al. 1997). Wounded *S. lycopersicum* induce serine proteinase inhibitors Pin1 and Pin2 (Plunkett et al. 1982), and the wound signaling molecule methyl jasmonate also induces a cysteine proteinase inhibitor in *S. lycopersicum* (Bolter 1993). Colorado potato beetles exposed to induced potatoes develop proteases with insensitivity to cysteine inhibitors (Bolter and Jongsma 1995). Perhaps the inhibition of transcription of *pin1* and *pin2* is an alternative mechanism to developing new proteases insensitive to constitutively expressed midgut proteases.

We have begun a characterization of a component of CPB regurgitant that is inhibitory to two defense genes in *S. lycopersicum*. It is unclear whether this is similar to the compound that enhances the defensive enzymes peroxidase and polyphenol oxidase in potato (Kruzmane et al. 2002) because it was not characterized. Further work is required to elucidate the components of CPB regurgitant that are involved in the plant/insect interaction.

pin1 and *pin2* have been used extensively as marker genes for the examination of wound-induced signal transduction. In tomato, systemin is induced by wounding, leading to the biosynthesis and then perception of jasmonic acid, and results in the expression of *pin1* and *pin2* (Schilmiller and Howe 2005). Wound-induced genes have been subdivided into early- and late-expressed genes, with both *pin1* and *pin2* categorized as late wound-induced

genes (Ryan 2000). It would be valuable to determine where the regurgitant interacts with this signaling cascade. Is the down-regulation specific to *pin1* and *pin2* or are all late-induced genes also repressed? When regurgitant from *M. sexta* was added to wounded leaves of *N. attenuata*, jasmonic acid was induced to higher levels than in "control" wounded leaves (Halitscke et al. 2001). In addition, regurgitant up regulated 51 genes, whereas 66 genes were down regulated when compared to leaves exposed only to wounding (Halitschke et al. 2003). If regurgitant affects tomato in a similar manner, then it acts early in the signal transduction pathway before jasmonic acid is induced. In addition, it would not just down-regulate wound-induced genes in the signal transduction pathway, but it would enhance other wound responsive genes. Experiments sorting out these questions in tomato and potato are underway.

Acknowledgement We are indebted to R. Bennett (USDA Agricultural Research Service) for care and handling of tomato plants and the CPB colony. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

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J Chem Ecol (2007) 33:1049–1064
DOI 10.1007/s10886-007-9281-6

Browse Quality in Quaking Aspen (*Populus tremuloides*): Effects of Genotype, Nutrients, Defoliation, and Coppicing

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Received: 11 September 2006 / Accepted: 12 March 2007 /
Published online: 3 April 2007
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Abstract The consequences of interactions among genetic, ontogenetic, and environmental factors for the quality of winter-dormant tissues as food for browsing herbivores is poorly understood. We conducted two sequential common garden studies to assess the impacts of intraspecific genetic variation, nutrient availability, prior defoliation, and ontogenetic stage on the chemical quality of winter-dormant tissue in quaking aspen (*Populus tremuloides* Michx.). In the first study, saplings of 12 aspen genotypes were grown under low and high soil nutrient conditions, with or without two successive seasons of defoliation. Quantity and quality of current year's twig growth were assessed. Twig production varied among genotypes and declined under low nutrient availability, but showed little response to prior defoliation. Chemical quality of sapling twigs varied substantially among genotypes, and in response to nutrient availability and prior defoliation. Overall, browse quality improved (nitrogen levels increased while phenolic glycoside and condensed tannin levels decreased) after defoliation. Growth and chemical variables exhibited low to moderate clonal repeatability (broad sense heritability) values. Our second study employed the same 12 genotypes, grown under high-nutrient conditions and with or without two seasons of defoliation. The trees were coppiced to produce root sprouts, which were chemically assessed 1 yr later. Rejuvenation via coppicing

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led to increased levels of nitrogen, phenolic glycosides (salicortin), and tannins in root sprouts, and the magnitude of change varied among aspen genotypes. Signatures of defoliation nearly 2 yr earlier persisted in terms of elevated levels of phenolic glycosides in root sprouts of previously defoliated trees. Aspen forests likely present browsing herbivores with chemically heterogeneous environments because of the interactions of genetic, ontogenetic, and environmental factors that vary over space and time.

Keywords Condensed tannins · Defoliation · Genetic variation · Heritability · Ontogeny · Phenolic glycosides · Phytochemistry · Plant–mammal interactions · Winter

Introduction

The quality of plant tissues as food for herbivores is determined by the complex interplay of plant genetics, ontogenetics, biotic factors such as prior feeding damage, and abiotic factors such as resource availability. The independent and interactive effects of these factors on the quality of foliage for herbivorous insects have been assessed in numerous studies (reviewed by Berenbaum and Zangerl 1992; Fritz and Simms 1992; Karban and Baldwin 1997; Agrawal et al. 1999; Nykanen and Koricheva 2004). Much less is known, however, about how these factors influence the quality of woody tissues for browsing mammals.

During winter periods in north temperate and boreal forests, herbaceous vegetation is of low nutritional quality because of senescence and is often inaccessible because of snow. Mammalian herbivores rely heavily on dormant woody plants for food (Bryant and Kuropat 1980). Browsing mammals typically feed on the current year's growth (CYG), including woody stems and buds. Such damage alters the quantity and quality of foliage produced in the following growing season, with attendant impacts on the performance of folivorous insects (Danell and Huss-Danell 1985; Den Herder et al. 2004; Hochwender et al. 2005; Valkama et al. 2005). The reciprocal interaction, i.e., the effects of defoliation on the subsequent quantity and quality of woody browse, has rarely been investigated.

Quaking aspen (*Populus tremuloides*) and European aspen (*Populus tremula*) are circumpolar in distribution, and are a major food source for browsing mammals in boreal, north temperate, and montane regions. In North America, aspen provides winter food for browsing mammals such as hare, beaver, deer, elk, and moose (Bryant 1981; Perala 1990; Romme et al. 1995). Aspen is also subject to regular and expansive insect outbreaks by species such as forest tent caterpillars, gypsy moths, and large aspen tortrix (Mattson et al. 1991). In the Great Lakes Region, for example, forest tent caterpillars defoliated nearly seven million hectares of forest in 2001. Much of this was aspen forest, where early summer defoliation rates averaged nearly 100% (Donaldson 2005). Quaking aspen thus provides an ideal experimental organism for addressing how winter browse quality is influenced by defoliation in preceding summers.

A characteristic form of asexual reproduction in aspens is the production of new sprouts from adventitious buds of roots, after death of the primary stem. "Coppicing" of aspen trees by natural agents (e.g., browsing mammals or fire) or by timber harvest typically causes prolific suckering, with densities of ramets reaching 30,000–100,000/ha (Zasada et al. 2001). Loss of competing apices in the crown and access to nutrients and growth-promoting cytokinins in the remaining roots leads to "rejuvenation" and rapid growth of root sprouts (Dickmann et al. 2001). In essence, coppicing restarts the ontogenetic clock of sprouts. The juvenile sprouts typically express high levels of chemical defenses, ostensibly as protection

against browsing mammals (Bryant 1981; Bryant et al. 1991; Bryant and Julkunen-Tiitto 1995; Boege and Marquis 2005). Then, as trees grow beyond the reach of mammalian herbivores, levels of chemical defenses typically decline. The effects of prior defoliation on the quality of winter-dormant sprouts and how these may vary among plant genotypes are unknown.

Two of the major determinants of tissue quality for browsing herbivores are nutrients (e.g., protein) and secondary metabolites (Lindroth 1989; Iason 2005). In aspen, the major secondary compounds are phenolics, including salicylate phenolic glycosides and condensed tannins (Lindroth and Hwang 1996). Concentrations of these compounds in aspen foliage are highly genetically variable, as well as differentially responsive to resource availability and prior feeding damage (Osier and Lindroth 2004, 2006; Stevens and Lindroth 2005). These compounds strongly influence interactions of *Populus* species with herbivores (Hemming and Lindroth 1995; Bailey et al. 2004, 2007; Donaldson and Lindroth 2007) and mediate ecosystem processes such as litter decomposition and nitrogen mineralization (Schweitzer et al. 2004; Madritch et al. 2006). Levels of these compounds also exhibit striking ontogenetic variation in aspen foliage, with high levels of phenolic glycosides and relatively low levels of tannins in young ramets, and the reverse in mature trees (Donaldson et al. 2006).

The research reported here had several objectives. First, we were interested in evaluating how aspen genetic variation, soil nutrient availability, prior defoliation, and interactions among those factors influence the quantity and quality of dormant winter browse in aspen. Plant defense theory suggests that levels of carbon-based secondary metabolites should increase in response to both relatively low soil nutrient availability (Herms and Mattson 1992) and prior defoliation (Bryant et al. 1991). Moreover, phenotypic plasticity in response to resource availability and defoliation is likely to vary among plant genotypes, especially in highly genetically variable species such as aspen (Perala 1990; Mitton and Grant 1996). We predicted that (1) current year's twig growth would be reduced under conditions of low nutrient availability or prior defoliation; (2) levels of nitrogen (an index of protein) would decline in twigs in response to low soil fertility or prior defoliation; (3) levels of phenolic glycosides, which are relatively nonplastic in response to environment (Osier and Lindroth 2004, 2006), would change little in response to experimental treatments; (4) levels of condensed tannins, which are highly plastic in response to environment (Osier and Lindroth 2004, 2006), would increase under low soil nutrients and prior defoliation; and (5) aspen genotypes would vary in response to nutrient availability and prior defoliation (significant gene × environment interactions).

Our second objective was to assess genetic variation in tissue quality of dormant juvenile sprouts after coppicing of saplings, and whether chemical profiles of the sprouts retain a signature of prior defoliation of the original (parent) ramet. No soil nutrient treatment was included in this study. We predicted that (1) sprouts would exhibit strong genetic variation in expression of chemical traits; (2) chemical profiles of sprouts would differ from those of parent ramets because of rejuvenation (increased concentrations of phenolic glycosides and decreased concentrations of tannins); and (3) prior defoliation to parent ramets would have no influence on chemical profiles of sprouts a year after removal of all aboveground biomass.

Methods and Materials

Experimental Design

We established a common garden of potted trees on the campus of the University of Wisconsin, Madison. The split-plot experimental design consisted of two soil nutrient

environments, two levels of defoliation (0% and 75%), and 12 aspen genotypes (details below). The defoliation rate of 75% corresponds to rates experienced by natural stands of aspen during outbreaks by forest tent caterpillars (Donaldson 2005). The soil nutrient and defoliation treatments were crossed at the whole plot level, with genotype incorporated at the subplot level. The 48 treatment combinations were replicated across 5 blocks for a total of 240 trees.

Genotypes

We micropropagated root material originally collected from 12 wild aspen genotypes growing in south-central Wisconsin. Micropropagation allows for the replication of many ramets from a single root source and decreases nongenetic effects (analogous to maternal effects) from source tissues (Wright 1976). Microsatellite DNA markers were used to verify that each genotype was unique (Cole and Lindroth, unpublished data).

Propagation

The micropropagates were planted outside into 5-l pots containing a 40:40:20 mix of sand/silt loam field soil/perlite in spring of 2001. All pots received Osmocote 3–4 mo slow release fertilizer (14:14:14 N/P/K + micronutrients) at a rate of 4.5 g/l of soil. In spring 2002, saplings (average height=1.1 m) were transplanted into the experimental garden comprised of 80-l pots. Each pot contained a 70:30 mixture of sand and silt loam field soil. High-nutrient pots received Osmocote 8–9 mo slow release fertilizer (18:6:12 N/P/K + micronutrients; 4.5 g/l) in the spring of both 2002 and 2003. Low-nutrient pots received no fertilizer.

Defoliation

Our defoliation treatment modeled an insect outbreak in both duration and intensity (Mattson et al. 1991; Parry et al. 2003). We severely defoliated experimental trees in two successive summers using both forest tent caterpillars and scissors. Insects were employed to provide natural cues (e.g., frass and saliva) that may be important to trigger tree responses to defoliation (Karban and Baldwin 1997; Havill and Raffa 1999), whereas scissors were used to ensure that all genotypes were damaged similarly regardless of their chemical quality (Stowe et al. 2000; Siemens et al. 2003). In early June of both 2002 and 2003, we exposed a subset of branches on each sapling in the defoliation treatment to forest tent caterpillar herbivory for 10 d. Scissors were then used to remove 75% of each leaf (for details see Stevens et al. 2007). Background levels of natural herbivory at our common garden were low.

Dormant Twig Collection

In March 2004, 9 mo after the second defoliation, we collected winter-dormant twig samples from 4 of the 5 experimental blocks (192 trees). We haphazardly selected five branches from each tree, and ensured that our sample included branches from lower, middle, and upper portions of the crown. The terminal leader was avoided. We measured the length and diameter of the CYG on each selected branch. Diameter was measured at the proximal end of the CYG using calipers. For chemical analyses, we collected the most distal 10-cm portion of each of the five branches per tree. For branches with less than 10 cm

of growth, the entire CYG was collected. If branches had dead tips, those portions were removed and discarded. In the few cases where the entire CYG was dead, length and diameter were measured but chemical analyses were not done.

Coppicing and Dormant Root Sprout Collection

In May 2004, 3 yr after the trees were planted outside, all of the high-nutrient trees were coppiced (severed at the soil surface with loppers). The coppiced trees were allowed to regenerate via root sprouting. In March 2005, we collected the terminal 10 cm of winter-dormant CYG from 3–4 sprouts per pot (1–2 sprouts per pot for 6 trees with little regeneration). Trees from all five experimental blocks of the high-nutrient treatment were sampled.

Phytochemical Analyses

Aspen samples consisting of multiple twigs or spouts per pot were freeze-dried and ground through a Wiley mill (no. 20 mesh). To improve homogeneity of samples, highly fibrous woody particles were removed via sifting through a no. 25 mesh screen. Samples were stored frozen (−20°C) until chemically analyzed.

We analyzed the tissue samples for chemical constituents likely to influence the feeding preferences of browsing mammals. These included nitrogen (an index of protein), phenolic glycosides (salicortin and tremulacin, which typically comprise >95% of the phenolic glycoside pool; Lindroth and Hwang 1996), and condensed tannins. Total nitrogen concentrations were determined with an FP452 LECO (St. Joseph, MI, USA) elemental analyzer. Glycine *p*-toluenesulfonic acid was used as a standard. Phenolic glycosides were quantified by high-performance thin layer chromatography using salicortin and tremulacin purified from aspen as standards (Lindroth et al. 1993). Quantification was effected on the basis of peak heights, and peak identity was confirmed via UV spectral scanning. Condensed tannins were extracted from tissues in 70% acetone (with 10 mM ascorbic acid, 4°C) and quantified by the butanol–HCl assay (Porter et al. 1986). Purified aspen condensed tannins (Hagerman and Butler 1980) served as the standard.

Statistical Analyses

Differences in sapling growth and in concentrations of chemical constituents in CYG among aspen genotypes, and between nutrient and defoliation treatments, were assessed in a three-factor split-plot analysis of variance, using JMP version 5.0.1a. Defoliation and nutrient treatments were whole plots (crossed), and sapling genotype was analyzed as a subplot effect nested within whole plots. In separate analyses, the effects of "ontogenetic stage" (i.e., sapling vs. postcoppice root sprouts), genotype, and defoliation on chemical constituents were assessed using PROC MIXED in SAS. The statistical model was similar to that described above, except that there was no nutrient treatment, and ontogenetic stage was analyzed as a repeated measure. Pairwise relationships among phytochemical variables (among saplings), as well as correlations between sapling and root sprout chemical composition, were assessed using simple correlation analyses performed with JMP. For the sapling–sprout correlations, we used data from the four experimental blocks for which we had paired measurements from "parent" and "offspring" (root sprout) plants.

In studies with multiple ramets of different genotypes, variance component analysis can be used to partition the variance in traits of interest into within- and between-genet

components (Falconer 1989). Because clonally derived plants of a single genotype have identical genetic material, differences among them can be attributed to environmental variation or sampling error. Differences among genets estimate V_G (total genetic variance), which is the sum of V_A (additive genetic variance, the component of genetic variance that responds to selection), nonadditive sources of genetic variance such as dominance and epistasis, and maternal effects. The proportion of total phenotypic variance (V_P) that is genetically based is known as broad-sense heritability ($H^2 = V_G/V_P$). When clonally derived plants are the subject of research, the term "clonal repeatability" is preferred in lieu of "broad-sense heritability" (Falconer 1989). Clonal repeatability is an indirect index (and upper bound) of V_A (Mitchell 2004). We calculated clonal repeatabilities as described by Falconer (1989). Standard errors for repeatability values were estimated following Becker (1984).

Results

Growth indices differed among aspen genotypes and in relation to environmental factors. Similarly, chemical constituents differed among aspen genotypes, and in relation to both environmental and ontogenetic factors. In the following text, descriptions of changes among treatment factors are provided in relative, not absolute, terms, unless noted otherwise.

Genetic and Environmental Effects on Winter-dormant, Sapling Twigs

Current year growth (i.e., twig production) varied among aspen genotypes and in response to nutrient availability, but only weakly in response to prior defoliation. The length of CYG twigs varied by 2.6-fold among genotypes (averaged across nutrient and defoliation treatments; Fig. 1). Twig length increased an average of 76% in high-nutrient trees relative to low-nutrient trees, and genotypes responded differently to changes in nutrient availability (significant genotype × nutrient effect). Twig length did not change significantly, however, in response to defoliation. The diameter of CYG twigs varied by 2.1-fold among genotypes and increased 58% in high-nutrient relative to low-nutrient trees (Fig. 1). Twig diameter declined by 10% in defoliated trees (a marginally significant response). When we incorporated both twig length and diameter into a metric of total twig production (calculated as diameter^2 × length), we found strong effects of genotype, nutrient availability, and genotype × nutrients, but no significant effect of defoliation (data not shown).

Chemical composition of CYG twigs also varied among genotypes and in response to nutrient and defoliation treatments. Nitrogen levels differed among genotypes, especially under high-nutrient conditions (Fig. 2; significant genotype × nutrient interaction). Nitrogen levels averaged 73% higher in aspen twigs under high-nutrient, relative to low-nutrient conditions. Prior defoliation influenced twig nitrogen concentrations, but differently so for the two nutrient treatments (significant nutrient × defoliation effect). Under low-nutrient conditions, defoliation led to a similar increase (29%) in nitrogen levels across genotypes. Under high-nutrient conditions, however, defoliation slightly increased, slightly decreased, or did not alter nitrogen concentrations in aspen genotypes.

Phenolic glycoside concentrations varied markedly among genotypes, but responded less strongly to environmental factors. Levels of salicortin in twig tissues increased slightly (8%) in high-nutrient relative to low-nutrient conditions, with some genotypes responding more strongly than others (Fig. 2; marginally significant genotype × nutrient interaction). Responses to defoliation also varied among genotypes, and included increases, decreases,

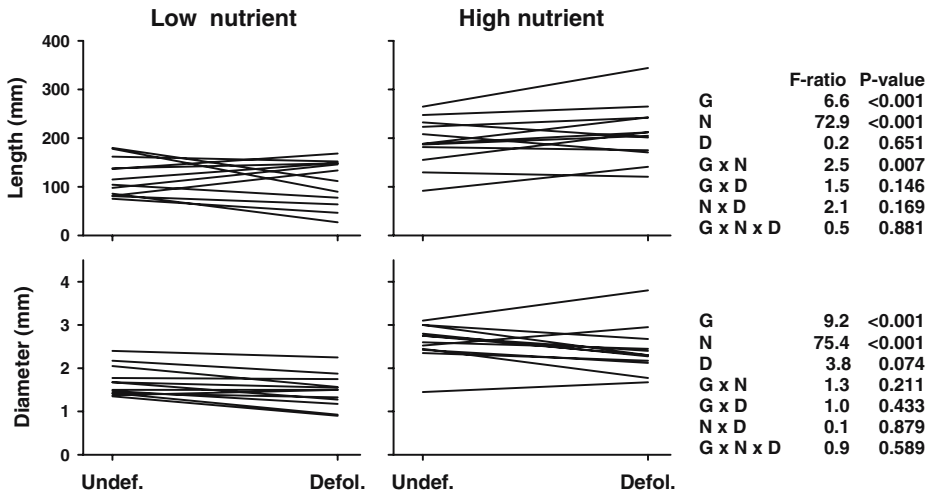


Fig. 1 Genetic and environmental effects on CYG of terminal branches (twigs) in quaking aspen saplings. Norm of reaction plots reveal responses for branch length and diameter after two successive seasons of 75% defoliation. Each line represents the mean response (N=4 replicate trees) of a single aspen genotype in the undefoliated vs. defoliated condition, under low and high soil nutrient availability. Summary statistics are provided to the right of the figure; G = genotype, N = nutrient treatment, D = defoliation treatment

and no change in concentrations (significant genotype × defoliation interaction). Levels of tremulacin showed a more pronounced increase (19%) under high-nutrient conditions, and again some genotypes responded more strongly than others (Fig. 2; significant genotype × nutrient interaction). With the exception of one genotype, tremulacin concentrations declined (an average of 15%) in response to prior defoliation.

As was the case for phenolic glycosides, condensed tannin concentrations varied most among genotypes and less in response to environment (Fig. 2). Levels varied an average of 1.6-fold among genotypes, and this variance was greater under high-nutrient than low-nutrient conditions (significant genotype × nutrient interaction). The marginally significant genotype × defoliation interaction reflects the fact that most genotypes exhibited slight declines in tannin levels in response to defoliation, whereas a few showed small increases or no change.

Genetic and environmental factors influenced sapling tissue quality in a similar manner for several chemical constituents (Table 1). Concentrations of both salicortin and tremulacin were positively correlated with concentrations of nitrogen. As expected, levels of the two phenolic glycosides were strongly and positively correlated with each other. Concentrations of condensed tannins were weakly and positively correlated with concentrations of tremulacin.

Clonal Repeatability of Sapling Twig Growth and Chemistry Variables

Both twig length and diameter exhibited moderately high clonal repeatabilities (Table 2), indicating a substantial genetic component in expression of those traits. The genetic component of variance in twig chemical characteristics varied from low to moderately high, even between structurally similar constituents (i.e., salicortin and tremulacin).

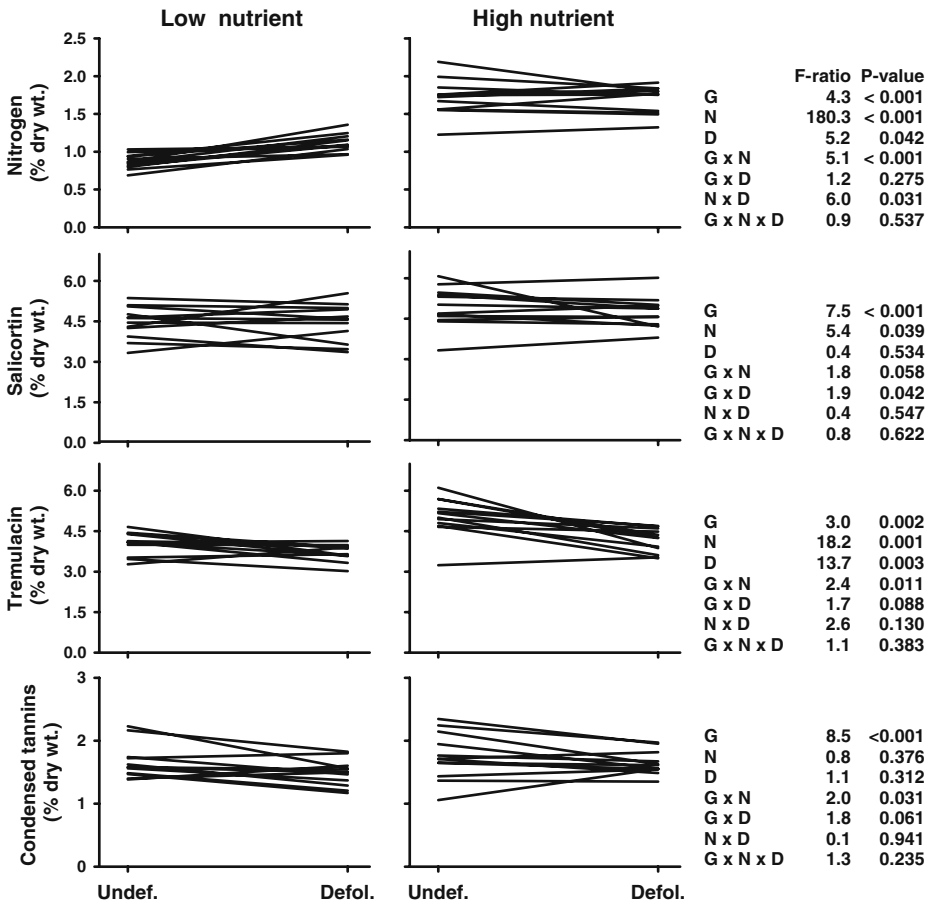


Fig. 2 Genetic and environmental effects on chemical composition of winter-dormant twigs in quaking aspen saplings. Norm of reaction plots reveal responses for nitrogen, salicortin, tremulacin, and condensed tannin concentrations after two successive seasons of 75% defoliation. Each line represents the mean response (N=4 replicate trees) of a single aspen genotype in the undefoliated vs. defoliated condition, under low and high soil nutrient availability. Summary statistics are provided to the right of the figure; G = genotype, N = nutrient treatment, D = defoliation treatment

Table 1 Correlation matrix (Pearson) for browse quality of dormant aspen saplings

	Nitrogen	Salicortin	Tremulacin
Salicortin	0.179*		
Tremulacin	0.352***	0.619***	
Condensed tannins	0.081	0.124	0.146*

*0.01<P<0.05

**0.001<P<0.01

***P<0.001.

Table 2 Clonal repeatability for dormant aspen twig productivity (CYG) and chemistry variables (SE = standard error)

Variable	Clonal Repeatability	SE
Twig length	0.318	0.045
Twig diameter	0.406	0.044
Nitrogen	0.214	0.044
Salicortin	0.353	0.045
Tremulacin	0.141	0.042
Condensed tannins	0.386	0.044

Genetic, Ontogenetic, and Defoliation Effects on Winter-dormant Root Sprouts

The chemical composition of dormant root sprouts shifted from that of parent saplings (i.e., as a function of ontogenetic stage), and differed both among genotypes and in response to defoliation history of the saplings. Nitrogen levels increased slightly (7%) in root sprouts, relative to sapling twigs, and genotypes varied in their magnitude of response (significant genotype × ontogenetic stage interaction; Fig. 3). Prior defoliation of aspen saplings did not significantly alter nitrogen levels in root sprouts. In general, nitrogen levels varied more among genotypes than in response to either coppicing or prior defoliation.

Phenolic glycoside concentrations exhibited strong genetic and ontogenetic effects, as well as interactions between those factors and defoliation (Fig. 3). Rejuvenation increased salicortin concentrations by 49 to 153% among aspen genotypes (significant genotype × stage interaction). Prior defoliation affected salicortin levels differently among genotypes (significant genotype × defoliation interaction). Defoliation had little effect on salicortin levels in saplings, but slightly increased (8%) levels in root sprouts (significant stage × defoliation effect). Similar to salicortin, tremulacin levels varied among genotypes, and differently so for the two ontogenetic stages (significant genotype × stage effect; Fig. 3). Contrary to the pattern exhibited by salicortin, however, average tremulacin levels were similar between rejuvenated root sprouts and saplings. Moreover, in response to prior defoliation, levels of tremulacin decreased (an average of 18%) in saplings, but increased slightly (an average of 5%) in root sprouts (significant stage × defoliation effect).

Condensed tannin concentrations varied among genotypes and ontogenetic stages, but not in response to prior defoliation, and showed relatively few interactive effects (Fig. 3). Rejuvenation stimulated an increase in tannin concentrations, ranging from 5 to 72% among genotypes (significant genotype × stage interaction).

Correlation analyses revealed surprisingly few significant relationships between parent sapling and root sprout chemical constituents (Table 3). We found positive correlations between parents and sprouts for nitrogen (marginally significant) and salicortin, but only in trees that had previously been defoliated.

Discussion

Although much research has addressed the interactive effects of genetics, ontogenetics, resource availability, and herbivore damage on the quality of foliar tissues, remarkably little work has evaluated the consequences of such interactions for the quality of winter-dormant woody tissues. To our knowledge, this is the first attempt to address all of those factors in a

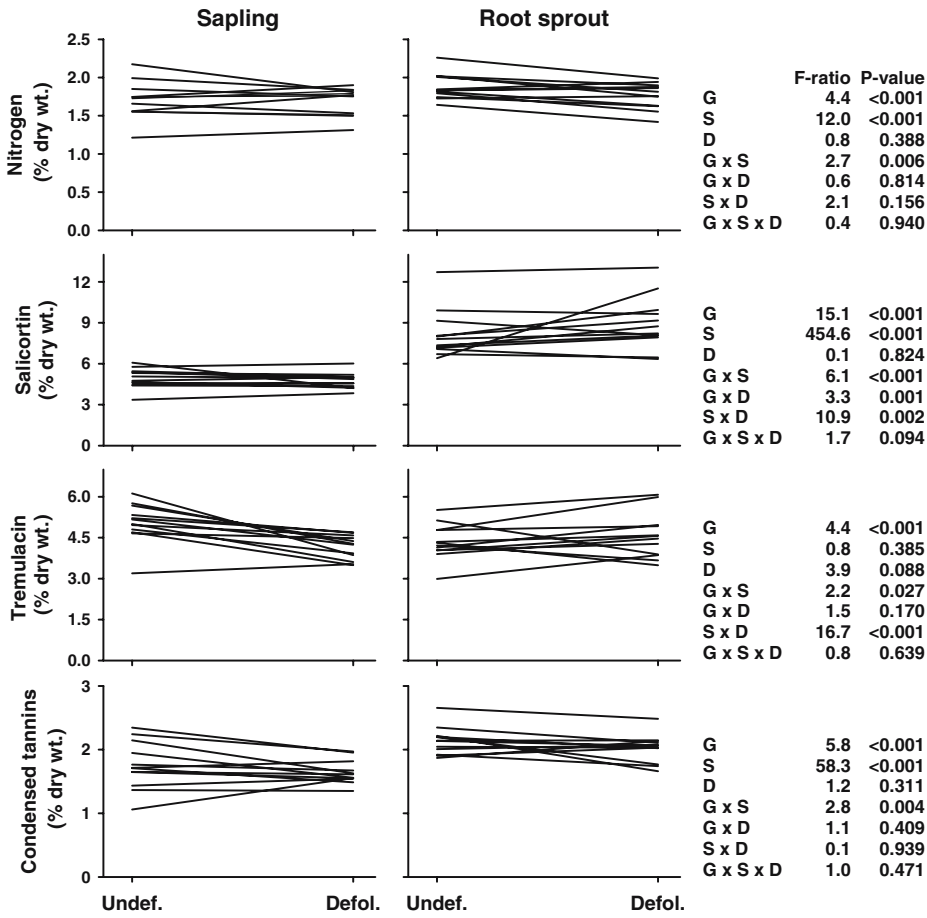


Fig. 3 Genetic, ontogenetic, and environmental effects on chemical composition of winter-dormant twigs in quaking aspen saplings and root sprouts. Norm of reaction plots reveal responses for nitrogen, salicortin, tremulacin, and condensed tannin concentrations after two successive seasons of 75% defoliation. Note the difference in scale for salicortin in Fig. 3 vs. Fig. 2. Each line represents the mean response of a single aspen genotype, as either saplings (N=4) or root sprouts (N=5), in the undefoliated vs. defoliated condition. Summary statistics are provided to the right of the figure; G = genotype, S = ontogenetic stage, D = defoliation treatment

Table 3 Correlations (Pearson) between sapling and root sprout chemistry

Variable	Undefoliated Trees		Defoliated Trees	
	r	*P*	*r*	*P*
Nitrogen	0.212	0.201	0.317	0.052
Salicortin	−0.095	0.570	0.399	0.013
Tremulacin	0.218	0.189	−0.070	0.678
Condensed tannins	0.036	0.828	0.089	0.605

single study. Here we document that for aspen, the production and chemical composition of dormant woody tissue is strongly influenced by a complex of independent and interacting genetic, ontogenetic, and environmental factors. The resulting temporal and spatial variation in food quality likely holds implications for the ecology and evolution of plant–mammal interactions.

Total current year twig production declined substantially under low-nutrient conditions, but weakly if at all in response to two successive seasons of heavy defoliation. This finding suggests that the availability of winter-dormant browse to vertebrate herbivores may not be significantly reduced in years after extensive defoliation by outbreak species such as forest tent caterpillars and large aspen tortrix. Moreover, growth responses to defoliation did not differ among aspen genotypes. This result is similar to those of a larger study (of which these trees were a part), which documented little genotypic variation in allocation of biomass to stem (aboveground woody) tissues, in response to defoliation of high-nutrient trees (Stevens and Lindroth, unpublished data). Finally, moderately high clonal repeatability values indicate that CYG twig production may show a strong response to selection, assuming no constraints beause of genetic correlations with other factors influencing fitness.

Nitrogen concentrations of dormant twigs increased in response to high-nutrient conditions and in response to prior defoliation in low-nutrient trees. The latter increase was contrary to our expectations; heavy defoliation typically causes fine root mortality and reductions in plant nitrogen concentrations (Bryant et al. 1991). These results suggest that defoliation may elicit changes in the sites of overwinter storage of nitrogen-containing compounds. The primary form of reduced nitrogen storage in perennating tissues of plants is vegetative storage proteins. In *Populus*, the major form of storage proteins is bark storage proteins, which accumulate during autumn senescence in tissues such as bark, wood, and roots (Cooke and Weih 2005). Extensive defoliation may shift allocation of bark storage proteins from roots and primary stems to CYG tissues, thereby elevating concentrations of nitrogen.

Levels of phenolic glycosides varied substantially among aspen genotypes, increased slightly to moderately under high-nutrient conditions, and exhibited mixed responses (salicortin) or declines (tremulacin) after defoliation. This pattern of substantial variation among genotypes, coupled with relatively small responses to resource availability and prior damage, is similar to that observed in previous studies of both rapid and delayed induced resistance in aspen foliage (Osier and Lindroth 2001, 2004). Overall, concentrations of phenolic glycosides in winter-dormant twigs were similar to those in leaf tissues of trees growing in the same common garden (Stevens et al. 2007) and in comparably aged trees (Donaldson and Lindroth 2007).

Condensed tannin concentrations also showed substantial genotypic variation, coupled with minimal and genotype-dependent responses to nutrient availability and defoliation. These latter, environmentally mediated changes were different from what we had predicted. Previous studies of aspen foliage have consistently demonstrated both substantial decreases in tannin concentrations when plants are grown in nutrient-rich environments (Bryant et al. 1987; Osier and Lindroth 2001, 2004, 2006; Stevens and Lindroth 2005; Donaldson and Lindroth 2007) and strong short- and long-term induction in response to defoliation (Osier and Lindroth 2001, 2004; Stevens and Lindroth 2005). This disparity in expression profiles between winter-dormant wood and summer leaf tissues may indicate differences in both the cost of metabolite production and the risk of tissue damage between the two seasons. Finally, overall levels of tannins in winter-dormant twigs were low in comparison with levels in foliage of comparably aged trees (Donaldson and Lindroth 2007; Stevens et al. 2007).

Although sapling aspen showed substantial genetic variation in terms of current year twig production and tissue chemistry, the genotypes tended to respond similarly to the

defoliation treatment imposed (i.e., relatively few genotype × defoliation interactions). Thus, phenotypic plasticity of woody tissue response to foliar damage may be constrained in aspen, and expressed more strongly in response to other environmental factors, such as resource availability. Such patterns have been observed for both short- and long-term induction responses in foliage (Osier and Lindroth 2001, 2004).

In summary, the general effect of prior, extensive defoliation was to not alter the quantity, but increase the quality, of winter-dormant twigs in sapling aspen. In this unique case of induced susceptibility, damage to one form of tissues (leaves), by one set of herbivores (outbreak insects), during the growing season, is likely to influence susceptibility of another set of tissues (CYG twigs), to another set of herbivores (browsing mammals), during winter dormancy. Whether preferences of and damage imposed by browsing herbivores in the field actually do change after major summer defoliation events remains unknown. We are aware of only one study that evaluated the impact of defoliation on subsequent mammal browsing. Hjältén et al. (1994) found that voles preferred previously defoliated birch seedlings over controls, despite the fact that defoliation did not significantly affect phenolic concentrations in birch shoots.

Estimates of heritability for quantitative traits indicate the proportion of phenotypic variance that is genetically determined. Heritability values calculated for concentrations of secondary metabolites in other experimental systems are highly variable, spanning the range from 0 to 1 (Berenbaum and Zangerl 1992; Laitinen et al. 2005). In this study, broad-sense heritability (clonal repeatability) values for secondary metabolites were low to moderate. Particularly low values (e.g., as for tremulacin) may reflect previous strong selection for the trait, and subsequent loss of genetic variation (Falconer 1989). Our value for tannins was similar to that reported by Laitinen et al. (2005) for birch condensed tannins, whereas our value for salicortin was similar to that reported by Orians et al. (1993) for the same compound in *Salix*. In contrast, Stevens and Lindroth (2005) and Donaldson and Lindroth (2007) reported high values (>0.70) of clonal repeatability for foliar concentrations of tannins and phenolic glycosides in aspen. This disparity may reflect a difference in the heritability of woody vs. foliar chemical constituents. Or, it may simply highlight the fact that estimates of heritability can vary greatly among habitats and studies. Because no general rules exist for extrapolating among environments (Pigliucci 2005), comparisons among studies must be interpreted cautiously.

During plant development, both the risks of herbivore damage and the costs of antiherbivore defenses are likely to change, leading to ontogenetic variation in the expression of defense traits (Boege and Marquis 2005). In general, levels of secondary metabolites tend to be higher in juvenile than in adult tissues (Bryant, 1981; Bryant and Julkunen-Tiitto 1995; Laitinen et al. 2005). Few studies, however, have evaluated genetic variation in defense traits across multiple developmental stages for plants growing in standardized environments (e.g., common gardens). Rejuvenation of biochemical and physiological characteristics in plant tissues is of particular ecological and evolutionary relevance in aspen, where damage to individual trees (ramets) because of browsing, logging, or fire typically elicits a dense flush of root sprout production.

We observed strong ontogenetic shifts in the chemistry of dormant root sprouts. Juvenile sprouts contained elevated levels of nitrogen, salicortin, and tannins, relative to the saplings from which they derived. Genotype × stage interactions were significant for all chemical variables, revealing that expression of genetic variation in defense differs among ontogenetic stages. That genotypes exhibit different ontogenetic trajectories (*sensu* Boege and Marquis 2005) explains why we observed few significant correlations in chemical traits between precoppice trees and their root sprouts. Somewhat surprisingly, signatures of

defoliation carried through to affect several chemical traits of sprouts, despite the facts that defoliation had occurred nearly 2 yr previously, and that all aboveground biomass had been removed 10 mo earlier. For nitrogen, the effects of prior defoliation on root sprouts mirrored those on saplings. For phenolic glycosides, however, concentrations generally increased in response to prior defoliation for root sprouts, but not for saplings.

Striking ontogenetic variation in biochemical and physiological function is a hallmark characteristic of *Populus* (Dickmann et al. 2001; Donaldson et al. 2006). Numerous studies relating the ontogeny of individual leaf development (within a season) to the biosynthesis, transport, and accumulation of secondary metabolites have employed *Populus* as a model system (e.g., Arnold and Schultz 2002; Kleiner et al. 2003; Orians 2005; Donaldson et al. 2006; Rehill et al. 2006). Additional studies have linked ontogenetic shifts in resistance (whole plant development) to variation in the distribution and abundance of herbivorous insects on poplars (Kearsley and Whitham 1989; Martinsen et al. 1998). Little research, however, has addressed ontogenetic variation in the chemical quality of woody tissues in *Populus*. Bryant (1981) showed that adventitious shoots of aspen produce increased amounts of extractable "resin" (chemical composition unknown), which deters feeding by snowshoe hares. Our results are consistent with that finding, and suggest that elevated levels of tannins and phenolic glycosides (salicortin) in dormant root sprouts may serve as deterrents to mammalian browsers. Indeed, consumption of aspen summer foliage and twigs by elk and porcupine is negatively related to concentrations of phenolic glycosides (Bailey et al. 2007; Diner et al., unpublished data; Wooley and Lindroth, unpublished data), and selection of *Populus* branches by beaver is inversely correlated with bark tannin levels (Bailey et al. 2004).

Given that levels of phenolic glycosides and tannins are strongly genetically determined in aspen, and confer defense against a variety of biotic and abiotic agents (Lindroth 2001), the question arises as to why genetic variation for these traits persists in natural populations. The conventional explanation is that the costs and benefits of chemical defense vary over time and space. Such appears to be the case with aspen. In young aspen, production of phenolic glycosides exacts a cost in terms of growth, particularly in resource-limited habitats (Osier and Lindroth 2006), whereas in older saplings, costs of chemical defense are greatest under favorable growing conditions (Stevens et al. 2007). How cost–benefit relationships are shaped by extensive root systems and clonal integration, particularly among aspen root sprouts after coppicing, is unknown.

In conclusion, aspen exhibits substantial genetic variation in expression of woody tissue chemistry, as has been established previously for aspen foliage. Browse quality is further influenced by ontogenetic stage, as well as by environmental factors such as soil nutrient availability and prior defoliation. Winter browsers, like summer defoliators, are likely to encounter a chemically heterogeneous environment because of the complex interplay of genetic, ontogenetic, and environmental factors that vary spatially and temporally in aspen forests.

Acknowledgments We thank Andy Vogelzang for field and laboratory assistance. This research was supported by National Science Foundation grant no. DEB-0074427 to RLL and an Environmental Protection Agency STAR Fellowship to MTS. Comments from two anonymous reviewers improved the manuscript.

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J Chem Ecol (2007) 33:1065–1081
DOI 10.1007/s10886-007-9287-0

Interspecific Variation of Floral Scent Composition in *Glochidion* and its Association with Host-specific Pollinating Seed Parasite (*Epicephala*)

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Received: 12 July 2006 / Accepted: 19 March 2007 /
Published online: 30 March 2007
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Abstract Trees of the genus *Glochidion* (Phyllanthaceae) are pollinated by females of *Epicephala* moths (Gracillariidae) whose larvae consume the seeds of the flowers that they pollinate. Each *Epicephala* moth species is specific locally to a single host species, although two to four *Glochidion* hosts often cooccur. To investigate the role of olfactory signals in maintaining the plant–moth specificity, we analyzed floral scent composition of five *Glochidion* species by using gas chromatography–mass spectrometry (GC-MS) and conducted Y-tube olfactometer bioassays with *Epicephala* moths and their host flowers. The GC-MS analysis showed that the floral scents of the five *Glochidion* species are dominated by (*R*)-(−)- and (*S*)-(+)-linalool, and (*E*)- and (*Z*)-β-ocimene, and that each species produces 6–20 compounds. Transformation of scent profiles by using chord-normalized expected species shared distances and analysis of the data with nonmetric multidimensional scaling showed that floral volatiles of cooccurring *Glochidion* species can be distinguished by relative chemical composition, especially that of minor compounds. The bioassay with pollinators of *Glochidion lanceolatum* and *Glochidion ruburm* further indicated that *Epicephala* moths are capable of discriminating their hosts by using floral odor. The results suggest that the floral scent of *Glochidion* is one of the important key signals that mediate the encounters of the species-specific partners in the *Glochidion–Epicephala* mutualism.

Keywords Bioassay · *Epicephala* · Floral scent · *Glochidion* · Obligate pollination mutualism · Species specificity

Introduction

Glochidion, a monoecious tree genus of the family Phyllanthaceae, consists of more than 300 species ranging from tropical Asia to Australia and Polynesia (Govaerts et al. 2000).

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All members of the genus have minute apetalous flowers that are pollinated at night exclusively by locally species-specific moths of the genus *Epicephala* (Fig. 1; Kato et al. 2003; Kawakita and Kato 2006). Each female moth lays an egg in the ovary after pollinating the flower with pollen carried on her proboscis. The hatched larva consumes some of the developing seeds while leaving others intact. Because *Glochidion* tree serves as the only larval host of the pollinator moth, neither partner can successfully reproduce in the absence of the other. Similar examples of obligate mutualisms between flowering plants and their seed-parasitic pollinators are classically known in the fig–fig wasp and yucca–yucca

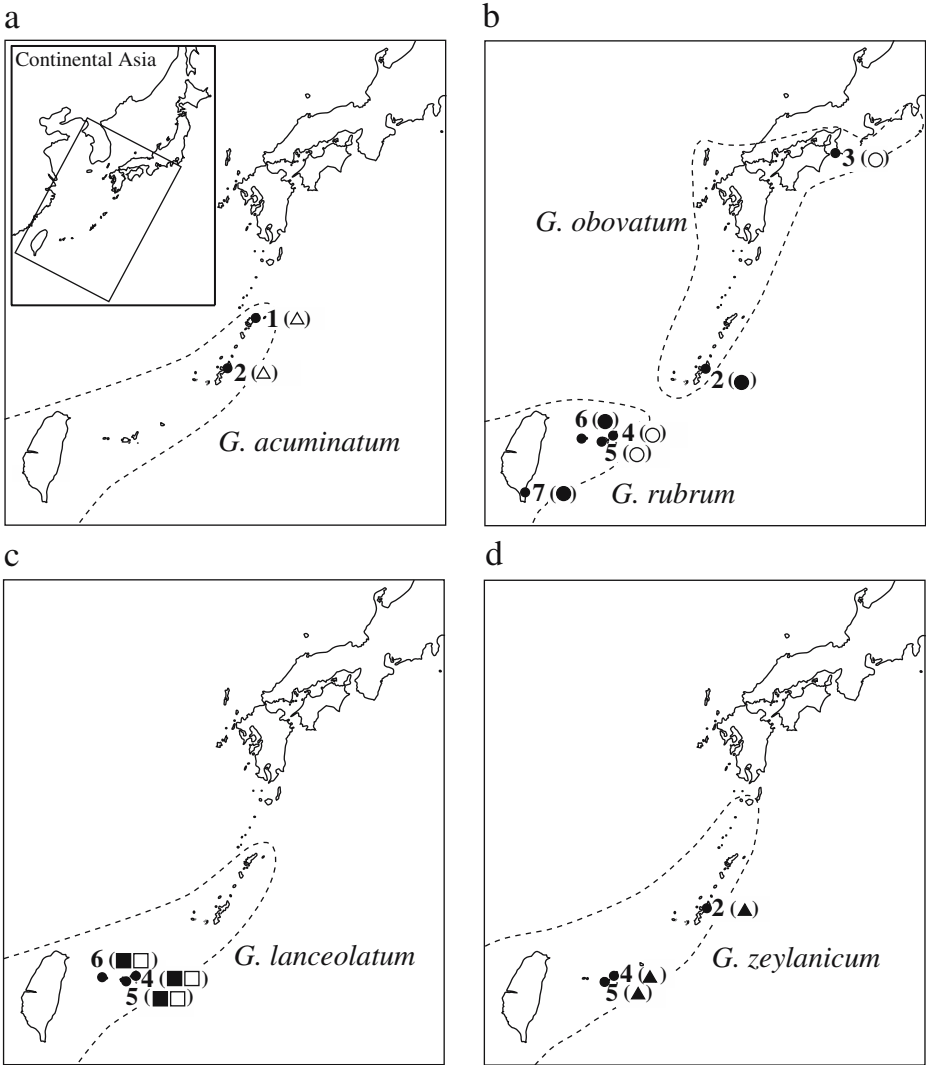


Fig. 1 Distribution of the five studied *Glochidion* species in western Japan and Taiwan. All but one (*G. obovatum*) species are widely distributed throughout Southeast Asia. Numbers indicate sampling localities corresponding to those in Table 1. Symbol(s) in parentheses indicate *Epicephala* moth species present at each host population, with different symbols representing different pollinator species

moth associations (Janzen 1979; Weiblen 2002; Pellmyr 2003). These mutualisms share various characteristics that are thought to have coevolved in the host and the pollinator, such as highly specialized floral structures and active pollinating behavior, which make them model systems for various studies of coevolutionary processes (Weiblen 2002; Cook and Rasplus 2003; Pellmyr 2003).

Among the most intriguing characteristics of these mutualisms are the high species diversity and species specificity of the lineages involved. For example, the figs comprise more than 750 species, and as many as 18 species occur sympatrically in the tropics (Corner 1965; Herre 1996). Meanwhile, the extremely high specificity of the pollinator to a single host suggests that a comparable number of wasp species also exists (Lopez-Vaamonde et al. 2002; Weiblen 2002; Molbo et al. 2003). Although less diverse, the Indo-Pacific genus *Glochidion* consists of more than 300 species, and up to 10 are known to grow sympatrically on the islands of Polynesia (e.g., Fiji Islands; Govaerts et al. 2000). Similarly, host specialization by the pollinators suggests that the pollinator moths are also highly diverse in this system (Kato et al. 2003; Kawakita and Kato 2006). The remarkable diversity and host-specificity of these interactions have sparked several analyses of the evolutionary dynamics of the interaction (Machado et al. 2001; Weiblen and Bush 2002; Kawakita et al. 2004; Weiblen 2004) and have provided opportunities for comparative studies on the ecological outcomes of mutualism (Herre 1989; Nefdt and Compton 1996; Herre and West 1997; Weiblen et al. 2001).

In cases where multiple plant species coexist in an obligate pollination mutualism, the maintenance of host specificity may be important in facilitating pollination by conspecific pollen and simultaneously allowing the pollinators to ensure larval nourishment by avoiding pollen incompatibility. Alternatively, the pollinators may be loyal to their hosts simply because their larvae suffer higher mortality on alternative hosts because of nutritional constraints, as is often observed in herbivorous insects (Via 1986; Singer et al. 1988; Traxler and Joern 1999; Bossart 2003; Forister 2004). Thus, understanding the mechanism by which the plant-pollinator specificity is maintained has important general implications for the role of specificity in these specialized pollination interactions, as well as the effects the mechanism may have on the evolutionary and ecological dynamics of mutualism.

In the fig–fig wasp mutualism, species specificity is maintained by responses of the pollinating wasps to specific fig volatile cues (Hossaert-McKey et al. 1994; Ware and Compton 1994; Song et al. 2001; Grison-Pigé et al. 2002a, b; Yokoyama 2003). For example, Grison-Pigé et al. (2002a) analyzed floral volatiles of four *Ficus* species and conducted biological tests of wasp stimulation by volatile samples to show that olfactory cues, especially floral volatiles, are often important signals that ensure the encounter of the two partners in this association. Because *Glochidion* flowers are small and inconspicuous (Fig. 2) compared with generally white or pale yellow flowers of most moth-pollinated plants (Proctor et al. 1996), it is unlikely that the nocturnal *Epicephala* moths detect the host plants by using only visual cues. Thus, olfactory cues are likely the predominant signal used by the moths to locate their hosts in the *Glochidion–Epicephala* mutualism.

In this study, we tested the following two hypotheses. If floral scents of *Glochidion* are used to detect host plants by moths, then (1) variations in floral scent composition should reflect differences in *Epicephala* moth species with which the plants are associated, and (2) *Epicephala* moth should be attracted to the floral scents of its host plant. We conducted two experiments to test these hypotheses. First, we identified the composition of the floral volatiles in five *Glochidion* species by using gas chromatography–mass spectrometry (GC-MS), and carried out a nonmetric multidimensional scaling (NMDS) analysis by using distance matrix obtained from floral scent profiles to investigate the differences in floral scents among the five

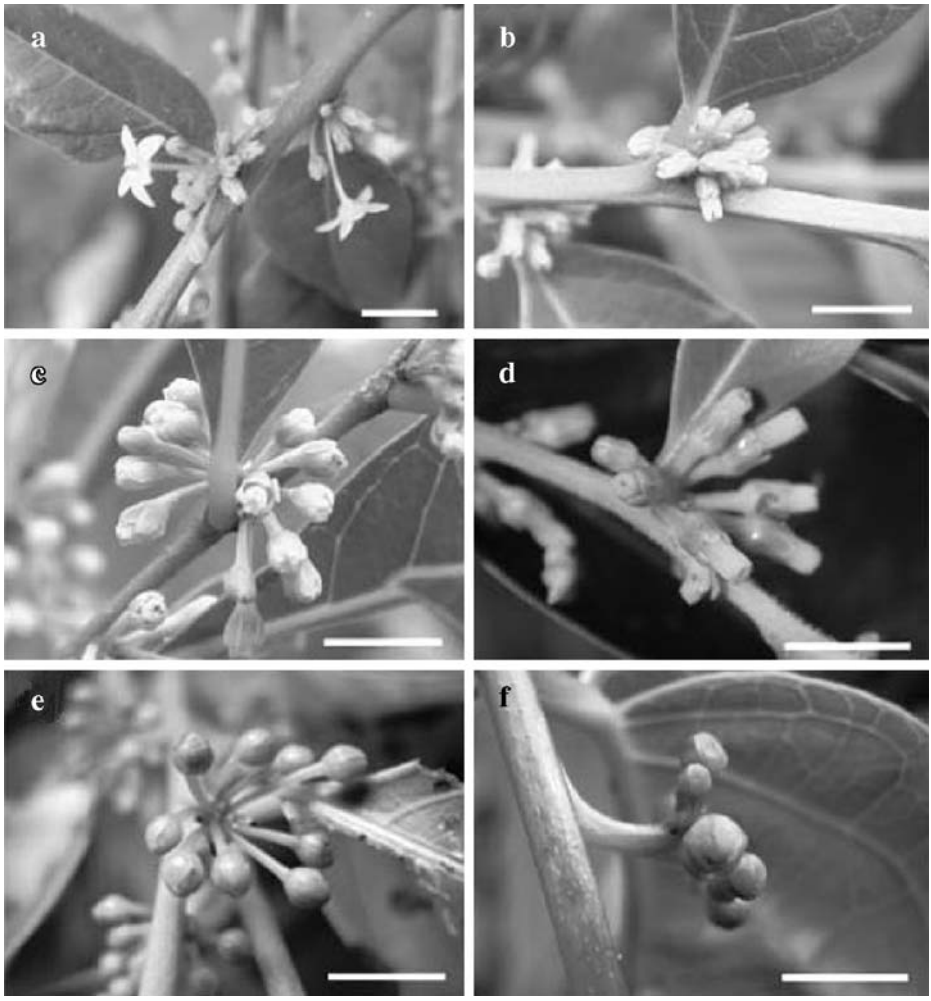


Fig. 2 Flowers of the five studied *Glochidion* species. **a** Male and female flowers of *G. acuminatum* aggregated at the axil. Female flowers of **b** *G. acuminatum*, **c** *G. obovatum*, **d** *G. rubrum*, **e** *G. lanceolatum*, and **f** *G. zeylanicum*. Bars=1 cm

Glochidion species. Second, we carried out a bioassay with a Y-tube olfactometer to determine whether *Epicephala* moths can detect their host plants by using floral scents.

Methods and Materials

Plants We studied five *Glochidion* species, *Glochidion acuminatum*, *Glochidion lanceolatum*, *Glochidion obovatum*, *Glochidion rubrum*, and *Glochidion zeylanicum*, that commonly occur in western Japan and Taiwan. *Glochidion obovatum* is distributed from temperate to subtropical regions of western mainland Japan and the Ryukyu Archipelago, whereas the other four species are more or less widely distributed throughout tropical Southeast Asia and as far north as the Ryukyu Archipelago (Fig. 1). They are common on forest edges, and two

to four species occur sympatrically at many of our study sites. *Glochidion* trees are monoecious and have male and female flowers aggregated on the axils (Fig. 2), the latter usually situated more proximally than the former. They have yellow male flowers with six perianths and less conspicuous female flowers that are more or less bud-like (Fig. 2). Flowers are open throughout the day and night and normally last about 1 wk. Flowers do not produce detectable nectar but are fragrant to the human nose during the evening, especially around 2000 hours. Although thrips are infrequently observed on male flowers during the day, insects other than *Epicephala* moths have rarely been observed on male or female flowers.

Among the five studied species, *G. lanceolatum* and *G. zeylanicum* are closely related (Kawakita et al. 2004) and share similar female flower morphologies (ovoid flower with styles almost completely covered with perianths; Fig. 2e, f). They are sympatric in the southern islands of Japan (Fig. 1). Similarly, *G. obovatum* and *G. rubrum* form another pair of closely related species (Kawakita et al. 2004), but their distributions are allopatric (Fig. 1). *Glochidion acuminatum* belongs to a distinct lineage and is equally related to the other four species.

Moths Six *Epicephala* moth species are associated with the five *Glochidion* species in our study region (Fig. 1; Kawakita and Kato 2006). *Glochidion acuminatum* and *G. zeylanicum* are each pollinated by a specie-specific *Epicephala* throughout its range in southern Japan. *G. obovatum* and *G. rubrum* are associated with two *Epicephala* species that are allopatric in distribution and pollinate the two *Glochidion* species at different parts of their ranges. Lastly, *G. lanceolatum* is pollinated by two *Epicephala* species that occur sympatrically in the southern Ryukyu Archipelago. Overall, each *Epicephala* pollinator is locally specific to a single host species, although each *Glochidion* species is pollinated by more than one moth species either in sympatry (*G. lanceolatum*) or in different parts of its range (*G. obovatum* and *G. rubrum*).

Collection and Identification of Floral Volatiles To test if the compositions of floral volatiles differs among *Glochidion* species, we collected floral volatiles by the headspace adsorption technique (Raguso and Pellmyr 1998) in the five *Glochidion* species. Study sites, dates, and sample sizes of the flowers used for headspace collection are shown in Fig. 1 and Table 1.

We used branches bearing both flowers and leaves for the collection of floral scents. For background, we used branches on the same plant bearing only the leaves. Branches were enclosed in low-density polyethylene bags. Air was purified by active charcoal and introduced into the bag through Tygon tubes with a minipump (flow rate, 300 ml/min; MP-2N, SIBATA, Tokyo, Japan). Volatiles in the bag were adsorbed by Tenax TA (50 mg; mesh 80–100; GL Sciences, Tokyo, Japan) stuffed into a glass tube (inner diam., 3 mm; length, 15 cm). We collected the volatiles for 120–200 min between 1900 and 2400 hours. The glass tube containing captured flower volatiles was sealed with glass beads, wrapped with aluminum foil, and stored at −30°C until used for analysis.

We eluted volatiles from the adsorbent with 2 ml of diethyl ether and added 5 μl of *n*-eicosane (1 mg/ml) as an internal standard. The eluate was carefully concentrated by N_2 flow to 25 μl and topped up with 25 μl of hexane. An aliquot (1 μl) of each sample was used for GC-MS (GC: GC-17A, MS: QP5050A; Shimadzu Inc., Tokyo, Japan). For GC, we used a DB-WAX capillary column (30 m×0.25 mm; film thickness, 250 μm; J&W Scientific, USA) and helium as the carrier gas. The injector was operated in the splitless mode for 1 min. Electron ionization mass spectra were obtained at an ionization voltage of 70 eV and a source temperature of 240°C. The oven temperature was programmed at 40°C

Table 1 Study sites, dates, and sample sizes of the headspace collection

Species	Study Sites[a]	Dates	Individuals[b]	No. of Flowers	
				Male	Female
G. acuminatum	Okinawa Island (2)	Apr 2004, May 2005	6	20–50	900–1000
	Amami-Oshima Island (1)	May 2004, May 2005	6 (5)		
G. lanceolatum	Ishigaki Island (4)	Sept 2004, Sept 2005	5 (3)	70–100	600–800
	Iriomote Island (5)	Sept 2005	2		
	Yonagini Island (6)	Sept 2004, Oct 2005	5 (2)		
G. obovatum	Tomogashima Island (3)	July 2004	3 (2)	10–40	200–400
	Okinawa Island (2)	June 2004, May 2005	3 (3)		
G. rubrum	Ishigaki Island (4)	Sept 2004, Oct 2005	11 (3)	20–50	200–300
	Iriomote Island (5)	Sept 2004, Sept 2005	3		
	Yonagini Island (6)	Sept 2004, Oct 2005	2 (2)		
	Nanren, Taiwan (7)	Aug 2004	1		
G. zeylanicum	Okinawa Island (2)	Apr, June 2004, May 2005	5 (3)	10–40	50–100
	Ishigaki Island (5)	Oct 2005	2 (2)		
	Iriomote Island (6)	Sept 2004, Sept 2005	7		

[a] Numbers in parentheses indicate locality numbers shown in Fig. 1.

[b] Numbers in parentheses indicate individuals resampled in 2006 for enantiomeric determination of linalool.

for 5 min, followed by an increase by 3°C/min to 100°C and 10°C/min to 200°C where it was held for 10 min. By comparing the volatiles emitted by branches with and without flowers, we isolated the volatiles emitted only from flowering branches and considered them the floral volatiles. For a preliminary identification of the compounds, we compared the fragments to those contained in the NIST 05 and NIST 05s libraries. We also calculated retention indices for all compounds by using *n*-alkane standards and compared them with those reported in previous studies (Davies 1990; Sagrero-Nieves et al. 1997; Gancel et al. 2002; Minh Tu et al. 2003). The identification of a subset of the compounds was further verified by using the retention indices and MS fragments of authentic compounds whenever possible. The percentage composition of each volatile compound was obtained by calculating its peak area as a percentage of the total peak area on gas chromatograms.

Determination of Linalool Enantiomers Because the above analysis indicated that the floral odor of *Glochidion* is often dominated by linalool, we performed an additional analysis by using a chiral column to discriminate the two enantiomers, (*R*)-(−)-linalool and (*S*)-(+)-linalool. We resampled floral scents from five trees for each of the five *Glochidion* species, which consisted of the same trees as used for the above analysis of floral scent profiles. Floral samples of *G. acuminatum* and *G. zeylanicum* were collected by headspace adsorption as described. For *G. lanceolatum*, *G. obovatum*, and *G. rubrum*; however, we sampled odor by removing the flowers from the branches in a 10-ml glass vial and pumping the air inside via a glass tube containing the Tenax TA adsorbent. This method does not allow for discriminating floral bouquet from background noise, but enables floral compounds to be adsorbed in a higher concentration, which facilitated enantiomeric determination and identification of other floral compounds produced in small amounts.

The volatiles were eluted and concentrated as described above except that 2 μl of *n*-hexadecane (1 mg/ml) were used as internal standard. The samples were analyzed with GC (GC-2010; Shimadzu Inc.) equipped with InertCap CHIRAMIX capillary column (30 m× 0.25 mm; film thickness 250 μm; GL Sciences). We used helium as the carrier gas. The

injector was operated in the splitless mode for 1 min. The oven temperature was programmed at 30°C for 5 min, followed by an increase by 1°C/min to 180°C, where it was held for 30 min. Before analyzing the floral samples, we analyzed authentic compounds of racemic linalool, (*R*)-(−)-linalool and (*S*)-(+)-linalool, including the *n*-hexadecane standard. Identification of the enantiomer was then conducted by comparing the retention time (standardized with *n*-hexadecane) of floral linalool with that of authentic compounds.

Statistical Analyses To examine differences of floral scents among the five *Glochidion* species, we performed a NMDS analysis based on relative GC peak area of floral compounds in each sample. For the NMDS, we used chord-normalized expected species shared (CNESS) distances to convert similarity between samples into metric indices. Chord-normalized expected species shared was originally designed to analyze faunistic or floristic similarity among biogeographic areas, and has been successfully incorporated in studies of floral volatiles (Jürgens and Dötterl 2004). Chord-normalized expected species shared distance is a dissimilarity index that reflects the expected number of species shared between a pair of samples in a random draw of *m* individuals, and ranges between zero and the square root of two. The advantage of CNESS is that sensitivity to changes in the abundance of dominant species (or dominant compounds in case of a floral bouquet) can be adjusted by altering the *m* parameter (CNESS*m*), thus emphasizing the importance of minor species (or compounds). Chord-normalized expected species shared distances were calculated by using the COMPAH program (Gallagher 1999). We rounded off percent value of each compound to two decimal points so that all detected compounds have nonzero values and multiplied them by 100 to convert to integers, as COMPAH does not appropriately handle decimals.

We determined the CNESS*m* having balanced influence to abundant and trace compounds in the floral samples by using the method described in Trueblood et al. (1994). We also used minimum and maximum CNESS*m* (m=1 and 9,999, respectively) that are most sensitive to dominant and minor compounds, respectively. NMDS analysis was performed with the SPSS 10.0J program to visualize the similarity between floral samples. When converting the distance matrix to a two-dimensional scatterplot in NMDS, a stress value is output, which represents how well or poorly a particular configuration fits the given data. The smaller the stress value, the better is the fit of the reproduced diagram to the distance matrix (Clarke 1993).

Bioassay To test whether *Epicephala* moths can detect their host plants by olfactory cues, we carried out a bioassay with a Y-tube olfactometer and the male and female moths reared from wild fruits of *G. lanceolatum* and *G. rubrum* that widely cooccur at our study sites (Fig. 1). We randomly collected fruits from 10 *G. lanceolatum* trees in September 2005 and October 2006 in Ishigaki Island, and 10 *G. rubrum* trees in October 2006 in Hateruma Island. They were kept in plastic containers under laboratory conditions (temperature 28.0°C, humidity 66.0–78.0%) until *Epicephala* larvae emerged from the fruits, at which point larval development is complete. After emergence, we removed the fruits from the containers to prevent possible negative effect of plant degradation on moth survival. Adult moths were used for the bioassay within 24 hr after eclosion. Intensive observation of adult moths behavior within the rearing containers indicated that the moths do not mate under such conditions (T. Okamoto, personal observations). Thus, the moths used for the test are most likely unmated individuals. Overall, we obtained 255 and 11 moths from *G. lanceolatum* and *G. rubrum* fruits, respectively.

For the bioassay, we connected the two arms of a Y-shaped glass tube (stem 13.5 cm, arm 11.0 cm, inner diam. 20.0 mm) via plastic tubes to polyethylene bags (48.0×34.0 cm), active charcoal, and mini pumps. Unidirectional airflow from the arms to the stem of the Y-tube was

purified by active charcoal and pumped into each bag, respectively (flow rate 20 ml/min). The experiment was done by using the following three pairs of odor bags: (1) a bag containing a flowering branch of a host (100–150 male and 500–550 female flowers) and an empty bag containing only air; (2) bags containing host branches with and without flowers; and (3) bags containing flowering branches of a host and a cooccurring nonhost. The first two experiments test for the roles of overall host fragrance and floral volatiles, respectively. The third experiment examines whether *Epicephala* moths can discriminate between a cooccurring pair of *Glochidion* using olfactory cues. *Epicephala* pollinators of *G. lanceolatum* were used in all three experiments. However, those of *G. rubrum* were used only in the third experiment because of low availability of moth specimens at our study populations.

Each moth was allowed 10 min to select either arm of the Y-tube in a dark condition. When the moth moved into an arm within 10 min and stayed for at least 30 sec, we considered the arm to have been selected. When a moth did not select either arm within a given time, we removed it and recorded that the moth had made no response. The bags connected to the two arms were randomly alternated in each test. After the experiments, moths were dried in silica gels for sex determination and species identification.

Identification of Epicephala Moths The *Epicephala* moths parasitizing *G. lanceolatum* fruits comprise two distinct, undescribed species (Kawakita and Kato 2006). Although male *Epicephala* can be determined by distinct genital morphology, there is subtle difference in female morphological characters. For this reason, we amplified a fragment of the mitochondrial cytochrome oxidase subunit I (COI) gene by using polymerase chain reaction (PCR) and species-specific primers to identify adult female moths used in the Y-tube test. We extracted genomic DNA from thoracic muscles with a standard phenol–chloroform method. We used the primers lanceolatum F (forward: 5′-CCC CCA CTT TCA TCA TTA ATA TTG CCC ATG CAG G-3′), lanceolatum R1 (reverse: 5′-TCC TCC TCC TGC AGG ATC AAA AAA T-3′), and lanceolatum R2 (reverse: 5′-ACC TCC ACC TGC TGG GTC AAA GAA G-3′). The two reverse primers were designed to amplify specifically either one of the two *Epicephala* species (A. Kawakita and T. Okamoto, unpublished data). Polymerase chain reaction conditions for amplifying the fragment containing the COI region were as follows: initial denaturation step at 94°C for 5 min; 30 cycles for 30 sec at 94°C, 30 sec at 55°C, and 1 min at 70°C; and final extension at 70°C for 7 min. All PCR amplifications were aided by Ex *Taq* DNA polymerase (TaKaRa, Otsu, Japan) and carried out on GeneAmp PCR System 9700 (PerkinElmer, Foster City, CA, USA). Presence/absence of successful PCR product was checked by electrophoresis on agarose gel.

Male moths pollinating *G. lanceolatum* used in the Y-tube bioassay were identified by examination of genitalia under a light microscope. For specimen preparation, we took off the apical end of the abdomen and boiled it in 10.0% potassium hydroxide for 5 min to remove abdominal tissue. We then washed it with distilled water and subsequently 100.0% ethanol to remove residual tissue fragments. After isolation of the genitalia, the specimen was mounted in Euparal (Waldeck, Münster, Germany) on a glass slide and observed under a microscope.

Results

Composition of Floral Volatiles Overall, we detected a total of 42 compounds in the headspace collections of the five *Glochidion* species. In each sample, 2 to 20 compounds

were found. We identified 69.0% of all compounds, whereas the remaining trace compounds had less than 1.0% peak area compared to that of the internal standard on GC-MS, and thus could not be identified. Most identified compounds were monoterpene and sesquiterpene compounds (Table 2). In all five species, floral samples were dominated by monoterpenes: (*R*)-(−)-linalool in *G. lanceolatum* (0.6–95.4% relative peak area) and *G. obovatum* (13.6–88.6%); (*E*)-β-ocimene in *G. acuminatum* (82.8–98.3%); (*E*)-β-ocimene and (*R*)-(−)-linalool in *G. rubrum* (0–71.3% and 0–90.7%, respectively); and (*Z*)-β-ocimene and (*S*)-(+)-linalool in *G. zeylanicum* (0.5–78.6% and 1.4–99.0%, respectively). Of the 42 compounds detected, 50.0% were shared by at least two species. Each *Glochidion* species had one to eight compounds that were unique. For example, *G. acuminatum* had β-myrcene and seven unidentified compounds that were not emitted by any other species. Most compounds unique to single species, except benzenoids, were produced in relatively small amounts, occupying 0.3–2.2% of the total peak area in each sample. A benzenoid, 2-phenoxyethanol, included in the *G. lanceolatum* samples occupied 3.9–71.9%, and phenylacetaldehyde and benzyl alcohol produced by *G. zeylanicum* occupied 0.0–87.0% and 0.0–53.8% of the total peak area, respectively.

Similarity of floral volatile profiles among individual samples was transformed by using CNESS distances and also subjected to the NMDS analysis. Scatterplots resulting from NMDS are shown (Fig. 3). The result of NMDS using CNESSm=1 (stress 0.17) indicates that floral samples were divided into three main species groups: (1) *G. acuminatum*; (2) *G. zeylanicum*; and (3) *G. lanceolatum*, *G. obovatum*, and *G. rubrum* (Fig. 3). These separations were likely caused by differences in the principal compounds included in floral scents of the three groups; *G. acuminatum* mainly contain (*E*)-β-ocimene, *G. zeylanicum* has (*S*)-(+)-linalool and/or (*Z*)-β-ocimene, and the other three *Glochidion* species have (*E*)-β-ocimene or (*R*)-(−)-linalool. The samples of *G. lanceolatum*, *G. obovatum*, and *G. rubrum* could not be distinguished from each other. When CNESSm=39 (stress 0.17) that is equally sensitive to dominant and trace compounds, the floral samples were divided into three main species groups as in CNESSm=1. In addition, the differences among the samples of *G. lanceolatum*, *G. obovatum*, and *G. rubrum* became more or less pronounced in this scatterplot. When trace compounds were maximally emphasized in the CNESS distance transformation (CNESSm=9,999, stress 0.18), the floral scents of the five species roughly grouped by species, although there were slight overlaps between the ranges of variation in the *G. lanceolatum*, *G. obovatum*, and *G. rubrum* samples. However, when only the samples of the above three species were included in the analysis, there was a clear separation between *G. lanceolatum* and the other two species, although the floral samples of *G. obovatum* and *G. rubrum* remained indistinguishable (results not shown).

Bioassay In the first two experiments that used the *Epicephala* pollinators of *G. lanceolatum*, the moths selected the odor of the flowering branch significantly more often than blank air or nonflowering branch (Table 3). In the third experiment, both the *Epicephala* pollinators of *G. lanceolatum* and *G. rubrum* selected the odor of their respective host significantly more often than that of the nonhost (Table 3). Overall, 5.0–10.0% of the moths tested did not make choices within the given 10 min in each experiment, and thus were excluded from the analysis. The tested moths consisted of 116 males and 139 females in *G. lanceolatum*, and 4 males and 7 females in *G. rubrum*. Inspection of male genital morphology and PCR amplification of the female moths indicated that all *G. lanceolatum* pollinators used in the bioassay belong to one of the two species that cooccur at our study sites.

Table 2 Floral scent compounds of the five *Glochidion* species expressed as percent relative peak area

Compound	RI[a]	*G. acuminatum*, *N*=12	*G. lanceolatum*, *N*=12	*G. obovatum*, *N*=6	*G. rubrum*, *N*=17	*G. zeylanicum*, *N*=14
Monoterpenes						
β-Myrcene[b]	1,150	0.3±0.49	–	–	–	–
(*S*)-(−)-Limonene[b]	1,181	–	6.61±7.58	–	2.61±4.20	–
(*Z*)-β-Ocimene	1,229	0.73±0.39	13.81±14.30	–	–	34.08±31.97
(*E*)-β-Ocimene[b]	1,249	94.34±5.64	–	6.38±14.05	25.66±23.38	–
150(100), 135(85), 107(28), 91(46), 79(41), 41(68)[c]	1,397	0.02±0.06	–	–	–	–
(*Z*)-Linalool oxide (furanoid)[b]	1,442	–	–	1.31±0.83	1.03±1.82	–
(*E*)-Linalool oxide (furanoid)[b]	1,476	–	1.00±2.06	8.96±7.52	2.59±2.65	–
(*R*)-(−)-Linalool[b]	1,565		53.33±34.80	73.19±28.96	45.88±34.61	
(*S*)-(+)-Linalool[b]	1,565	0.05±0.11	–	–	–	30.07±34.46
α-Terpineol[b]	1,697	–	–	0.45±0.91	0.11±0.31	–
(*Z*)-Linalool oxide (pyranoid)[b]	1,745	–	–	1.11±1.71	0.11±0.47	–
(*E*)-Linalool oxide (pyranoid)[b]	1,770	–	–	0.01±0.02	0.09±0.25	–
Sesquiterpene						
δ-Elemene[d]	1,553	0.07±0.13	–	1.47±3.27	0.37±0.83	–
β-Elemene[d]	1,581	1.05±1.83	–	2.67±2.09	7.39±13.65	–
(*E*)-β-Caryophyllen[b]	1,611	–	1.44±2.27	–	–	–
204(45), 189(100), 147(27), 133(64), 119(21), 105(46), 91(47), 81(35), 55(29), 41(56)[c]	1,665	0.14±0.25	–	0.40±0.63	1.76±4.20	–
Germacrene D[d]	1,696	–	–	–	1.83±5.78	–
α-Selinene[d]	1,704	–	–	–	1.27±2.11	–
δ-Selinene[d]	1,715	0.19±0.31	–	0.21±0.34	1.21±2.00	–
(*E*)-α-Farnesene[b]	1,744	1.58±2.69	0.13±0.44	–	–	–
β-Selinene[d]	1,745	0.19±0.28	–	0.55±0.81	0.79±1.83	–
γ-Elemene[d]	1,746	0.54±0.72	–	0.48±0.89	–	–
222(3), 204(38), 189(29), 161(21), 135(31), 95(27), 81(57), 55(32), 43(100)[c]	1,997	0.04±0.14	–	–	–	–
204(45), 189(99), 161(18), 147(31), 133(78), 105(54), 77(69), 55(60), 41(100)[c]	2,202	–	–	–	2.85±4.92	–
Irregular terpene						
(*E*)-Geranyl acetone[b]	1,855	–	1.30±2.34	–	–	3.41±4.26

Table 2 (continued)

Compound	RI[a]	*G. acuminatum*, *N*=12	*G. lanceolatum*, *N*=12	*G. obovatum*, *N*=6	*G. rubrum*, *N*=17	*G. zeylanicum*, *N*=14
Aliphatics						
Nonanal[b]	1,372	–	5.77±6.63	–	–	–
2-Ethylhexanol[b]	1,521	0.37±0.59	3.31±4.61	–	–	–
Benzenoids						
Benzaldehyde[b]	1,516	–	–	–	–	3.04±4.08
Phenylacetaldehyde[b]	1,633	–	–	–	–	18.53±28.43
Benzyl alcohol[b]	1,822	–	–	–	–	10.87±15.10
2-Phenoxyetanol[b]	1,880	–	12.13±20.70	–		–
Eugenol[b]	2,175	–	–	0.03±0.07	2.94±4.74	–
Miscellaneous						
(*Z*)-Jasmone[b]	1,942	0.02±0.05	–	0.02±0.04	0.62±0.92	–
Unidentified compounds						
Unidentified compound I[e]	1,452	0.24±0.83	–	–	–	–
110(43), 95(15), 84(15), 68(100), 55(21), 43(34)[c]	1,462	–	–	2.76±6.37	–	–
141(2), 125(2), 110(39), 95(12), 82(18), 68(100), 43(37)[c]	1,478	–	–	–	0.13±0.49	–
Unidentified compound II[e]	1,774	–	0.51±0.86	–	–	–
128(3), 115(1), 98(5), 83(32), 71(51), 57(26), 43(100)[c]	1,779	–	0.66±1.31	–	–	–
107(11), 96(36), 79(100), 67(49), 55(18), 41(53)[c]	1,782	0.01±0.03	–	–	–	–
220(2), 178(14), 149(9), 124(14), 108(14), 91(26), 79(28), 55(100), 41(48)[c]	1,995	0.06±0.10	–	–	–	–
168(1), 110(12), 82(14), 59(100), 41(15)[c]	1,955	0.03±0.05	–	–	–	–
152(82), 121(49), 105(53), 91(100), 67(97), 55(46), 43(95)[c]	2,012	0.04±0.08	–	–	–	–

[a] Retention indices relative to *n*-alkanes on the DB-WAX column.

[b] Identified based on mass spectrum and Kovats retention index (KI) of authentic compound.

[c] Ion fragments and their intensities are given for unidentified compounds.

[d] Identified by similarity of mass spectrum to those in the libraries and previously published KI index.

[e] Detected only by GC (undetectable peak in GC-MS).

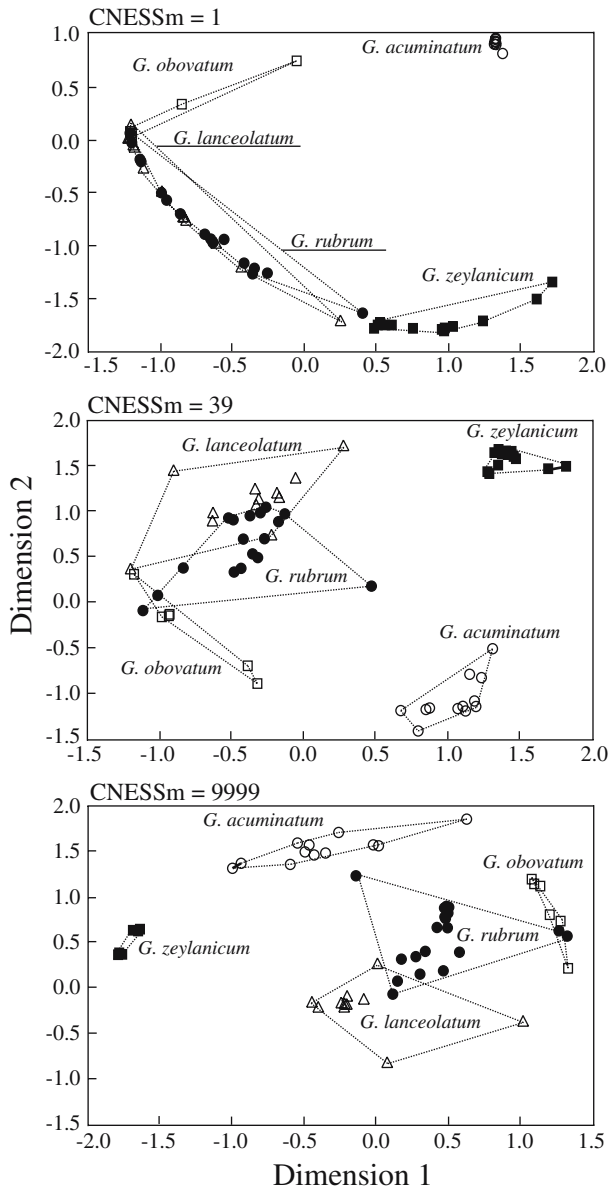


Fig. 3 Scatterplots resulting from the NMDS analysis of floral scents of the five *Glochidion* species

Discussion

Overall, 2 to 20 compounds were detected in each floral sample of *Glochidion*, and in total, 42 compounds were found. The 30 identified compounds are common to floral scents of many flowering plants (Knudsen et al. 1993). For example, monoterpenes, such as (*E*)- and (*Z*)-β-ocimene, (±)-linalool, linalool oxide, and eugenol are emitted by a wide range of flowering plants that are pollinated by various animals, such as moths (Knudsen and

Table 3 Results of Y-tube bioassay by using *Epicephala* moths pollinating *G. lanceolatum* and *G. rubrum*

Pollinator	Test Performed	Number of Moths	Host or Flower Selected	Otherwise Selected	Significance[a]
G. lanceolatum	Host vs. air	83	68	15	$P<0.001$
	Host vs. nonhost	76	59	17	$P<0.001$
	Flower vs. leaf	62	49	13	$P<0.001$
G. rubrum	Host vs. nonhost	10	8	2	$P<0.05$

[a] Binomial test

Tollsten 1993; Miyake et al. 1998; Raguso et al. 2003; Makholela and Manning 2006), butterflies (Andersson et al. 2002), beetles (Pellmyr et al. 1991; Jürgens and Dötterl 2004), bees (Gerlach and Schill 1991; Borg-Karlson et al. 1994), and bats (Pettersson et al. 2004). In addition, (±)-linalool, linalool oxide, and (*E*)-α-farnesene are attractive or at least shown to be electroantennographically positive to lepidopterans (Raguso et al. 1996; Hern and Dorn 1999; Bäckmann et al. 2001; Andersson and Dobson 2003). Most of these compounds were shared by the five *Glochidion* species, and floral scent of each species was not dominated by any compound unique to one species.

The result of CNESS-NMDS analysis based on CNESSm=1 indicated that the floral samples of the five *Glochidion* species can be grouped into three distinct types based on relative proportion of the dominant compounds, such as (*E*)-β-ocimene, (*Z*)-β-ocimene, and (±)-linalool (Fig. 3). This indicates that, at least for some species pairs, floral odors can be clearly discriminated based solely on dominant compounds. When principal and trace compounds were equally weighted in the CNESS distance calculation (CNESSm=39), the distinction between species became evident, although there were still overlaps among the samples of *G. lanceolatum*, *G. obovatum*, and *G. rubrum* (Fig. 3). Finally, when trace compounds were maximally emphasized in the analysis, the floral scents of *Glochidion* roughly grouped by species (Fig. 3). The floral samples of the two cooccurring species, *G. lanceolatum* and *G. rubrum*, were clearly separated when only the samples of these two species were included in the analysis (results not shown), indicating that the distinction between them was masked in the scatterplot shown in Fig. 3 because of inclusion of more dissimilar samples in the NMDS configuration. These results suggest the possibility that the presence of minor compounds and/or their amount relative to other compounds are also important in attracting specific *Epicephala* pollinators. However, a further study that uses electroantennographic detection analysis and field trapping with authentic chemicals is needed to test which compounds have the actual physiological activity to the specific pollinator of each species.

The floral scent of *G. acuminatum* was easily distinguished from that of the other four *Glochidion* species, as it was dominated by (*E*)-β-ocimene and contained a few minor compounds that were not emitted from the other four species, such as β-myrcene and an unidentified monoterpene (Table 2). *Glochidion obovatum* and *G. rubrum* had similar compositions of floral scent and did not have distinct proportions of either the principal or minor compounds (Table 2; Fig. 3), which possibly reflects phylogenetic relatedness (Kawakita et al. 2004). Although their floral scents could not be distinguished from each other (Fig. 3), they do not need to be distinct because their distributions do not overlap (Fig. 1) and the two hosts share the same two pollinators (Kawakita and Kato 2006). *Glochidion lanceolatum* and *G. zeylanicum* are also closely related; however, the results of CNESS-NMDS

indicated that floral scents of these could be distinguished based on both principal and trace compounds (Fig. 3). Such dissimilarity may be particularly important ecologically as the two *Glochidion* species have distinct pollinators and commonly occur in sympatry.

The Y-tube bioassay indicated that male and female pollinator *Epicephala* moths are attracted to the scents released from the host *Glochidion* trees, particularly those produced from the flowers (Table 3). These results are consistent with our prediction that olfactory cue is a key stimulus used by *Epicephala* moths to locate their host plants. Furthermore, the *Epicephala* pollinators of *G. lanceolatum* and *G. rubrum* were attracted to the odor of their respective host plants, indicating that *Epicephala* moths can discriminate between odors of the host and cooccurring nonhosts. Because the CNESS-NMDS analysis indicated that the floral scents of the two species were distinguishable based on minor compounds, the presence/absence of compounds unique to each species or characteristic proportions of relatively minor compounds may be the key decisive signal for moths to locate their host plants. The fact that both sexes of the pollinator were attracted further points to the possibility that host plants are used as mating sites because the males do not engage in pollination or oviposition, and the moths used in the test were unmated. Ideally, more *Epicephala* moths that pollinate *G. rubrum* need to be tested in the Y-tube bioassay, but these *Epicephala* moths occur at a very low density throughout our study range in the southern islands of Japan.

The differences in floral scents among *Glochidion* species that were found in this study may have resulted from a nonadaptive process but they can also have resulted from coevolution with *Epicephala* pollinators. Exploration of the extent to which floral scent of *Glochidion* is shaped by odor preferences in *Epicephala* pollinator needs further exploration by using dense sampling of plant and moth species and a robust phylogenetic hypotheses for the two groups. However, our results have implications for understanding the evolution of floral scent in the *Glochidion–Epicephala* mutualism. Within our study range, *G. obovatum* and *G. rubrum* share a pair of allopatric, phylogenetically distant *Epicephala* species that pollinate the two different *Glochidion* species within different populations (Fig. 1). The result of CNESS-NMDS analysis indicated that floral scents of *G. obovatum* and *G. rubrum* remain indistinguishable when blocked by the *Epicephala* species with which they interact (results not shown), indicating the absence of floral scent differentiation based on pollinator species. Furthermore, in several populations of *G. lanceolatum* in our study range, the plants host two divergent pollinator species, although only one of them was sampled in the present bioassay perhaps because of phenological difference between the two species. These observations suggest that floral scent differentiation does not necessarily occur when two pollinator species interact with a single host species. A similar lack of variation based on pollinator species is found in an ecologically similar yucca–yucca moth interaction (Svensson et al. 2005).

Overall, our study provides supportive evidence that floral scent of *Glochidion* is used as a signal by *Epicephala* moths to locate their specific host plants. The results provide a baseline for elucidating the mechanism that underlies the unusually high species specificity in the pollination mutualism between *Glochidion* and *Epicephala*. Furthermore, the *Glochidion–Epicephala* mutualism is a unique situation among other obligate pollination mutualisms in that the plant has separate male and female flowers, which may produce different odor blends to induce complex pollination behavior in the moths. Further study of the floral scents in *Glochidion–Epicephala* mutualism may provide additional insight into evolutionary and ecological aspects of the mutualisms between plants and their obligate seed-parasitic pollinators.

Acknowledgements We appreciate Y. Inui for providing the opportunity to conduct this study; T. Y. Chiang, T. W. Hsu, and S. C. Liu for assistance in the field in Taiwan; Y. Yamaoka and I. Shimizu for allowing us to use the GC and GC-MS; Y. Kumano and N. Fujiwara-Tsujii for technical advice in collecting and analyzing the floral scents; and the subject editor and anonymous reviewers for valuable comments on the manuscript. This work was supported by Grant-in-Aid for Scientific Research 15370012 from Japan Ministry of Education, Culture, Sports, Science and Technology (to M. K.) and by Grant for Basic Science Research Project from Sumitomo Foundation (to T. O.).

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J Chem Ecol (2007) 33:1083–1088
DOI 10.1007/s10886-007-9290-5

RAPID COMMUNICATION

The Influence of Tissue Handling on the Flavonoid Content of the Aquatic Plant *Posidonia oceanica*

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Received: 14 December 2006 / Revised: 12 March 2007 / Accepted: 19 March 2007 /
Published online: 3 April 2007
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Abstract In recent times, more and more studies have focused on flavonoids as biomarkers of environmental quality in aquatic plants, in particular, *Posidonia oceanica* (Linnaeus) Delile. It is therefore of interest to determine how different prehandling methods can affect flavonoid concentrations. The methods tested were (1) immediate extraction of fresh samples, (2) extraction after 48 hr chilling, (3) freeze-drying, and (4) oven drying. Chilling and freeze-drying considerably altered the quantity of flavonoids measured, but not their profile. The effect of oven drying was not significant. Chilling led to a loss of 57% of total (pro)anthocyanidins, 39% of total flavonols, and 48% of all simple flavonols (myricetin, quercetin, isorhamnetin, and kaempferol). Freeze-drying caused a loss of 71% of total (pro) anthocyanidins, 87% of total flavonols, and 95% of all simple flavonols.

Keywords Prehandling methods · Chilling · Freeze-drying · Oven-drying · *Posidonia oceanica* · Flavonoids · (pro)Anthocyanidins · Flavonols

Introduction

Marine Magnoliophyta are of increasing interest as bioindicators because of their worldwide distribution in the littoral zone, their longevity, and their potential to provide useful information on the evolution of environmental quality (Cannac et al. 2006). Many studies of phenolics have been carried out, in particular of flavonoids (Kaundun et al. 1998), as they have the potential to be reliable biomarkers of environmental quality (Cannac et al. 2006). Flavonoids are divided into 12 classes, according to the oxidation level of their central pyran ring. The most important and widespread are flavonols and (pro)anthocyanidins.

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Flavonoids have mostly been studied in terrestrial plants (Kaundun et al. 1998), whereas only a few studies have been performed on aquatic plants (Cannac et al. 2006).

Most studies indicate that, whenever possible, intact fresh plant material should be used in the analysis of secondary plant metabolites (Julkunen-Tiitto and Tahvanainen 1989). However, the high moisture content and increased enzymatic activity of fresh samples and the difficulty of repeating experiments may often invalidate and restrict the use of such material. When working on a large number of field samples collected from distant locations, it is necessary to prepare the material so that it can be stored for some time before analysis (Julkunen-Tiitto and Tahvanainen 1989).

Given the importance of secondary metabolites in plant defense, and because individual flavonoids exhibit different biological activities, proper methods for preservation of foliar material prior to chemical analysis are important (Orians 1995). Inappropriate methodologies may cause substantial changes in concentration that can lead to false interpretations. Many studies have highlighted the damages provoked by freeze-drying and oven drying on phenolic content in terrestrial plants (Julkunen-Tiitto and Tahvanainen 1989; Orians 1995; Lindroth and Koss 1996; Julkunen-Tiitto and Sorsa 2001). In these studies, the appropriate prehandling method depends upon the chemical species (Orians 1995), the part of the plant, and the plant considered (Julkunen-Tiitto and Tahvanainen 1989). Concerning aquatic plants, Steinberg (1986) demonstrated that freezing seems to cause changes in total phenolic compounds for *Sargassum* sp. (phaophyceae), but otherwise, no other prehandling study has been carried out.

Our purpose here was to verify to what extent different prehandling methods can alter flavonoid concentrations [total (pro)anthocyanidins and total and simple flavonols] for *Posidonia oceanica* (Linnaeus) Delile, the most widely studied species of marine Magnoliophyta in the Mediterranean (Cannac et al. 2006).

Methods and Materials

Plant Material *Posidonia oceanica* is organized into roots, rhizomes, and leaves, differentiated as follows (Cannac et al. 2006): (1) adult leaves consisting of a blade and a sheath, (2) intermediate leaves (length more than 50 mm without sheath), and (3) juveniles leaves (length less than 50 mm without sheath). Eighty *P. oceanica* foliar shoots were sampled by SCUBA diving on July 2006, in the Gulf of Calvi, Corsica (France, N 42° 34.811′, E 008°43.479′) at a depth of 15 m. Only blades were studied because phenolic concentrations are higher than in sheaths (Cannac et al. 2006).

Prehandling After sampling, shoots were kept cool in an icebox until their return to the laboratory (1-hr drive). All work was carried out in the dark. Blades were cleared of their epiphytes by gentle scratching with a glass blade, to alter the plant surface as little as possible and on a cool pack to avoid variations in temperature and to minimize chemical changes.

Samples were divided into four batches of 20 shoots, each of which underwent a different prehandling process, and within each batch, shoots were grouped in fours to obtain five replicates. The treatments were as follows: (1) immediate extraction of fresh samples, (2) chilling samples for 48 hr before extraction, (3) freeze-drying samples (FD 4-85, HETO ®) with prefreezing (−26°C) for 48 hr, and (4) oven-drying samples in a steam room (D-6450 Hanau, Heraeus) for 48 hr at 40°C.

Extraction Extraction and quantification of flavonoids [total (pro)anthocyanidins and total and simple flavonols] was based on the work of Kaundun et al. (1998). Thus, 1.6 g (dry weight) of *P. oceanica* was suspended in 100 ml of 2 M HCl. The solution was heated to 80°C in a water bath with reflux for 50 min with influxes of air every 10 min. The solution was left to cool for 30 min (solution A) and then, apart from 2 ml removed for measurement of (pro)anthocyanidins, was filtered through a no. 28 porosity filter. Flavonols were extracted from the filtered solution and from the filter wash liquid with 60 ml (twice) and then 40 ml (once) of diethyl ether. These different fractions were remixed and evaporated. The residue was kept in the freezer in the dark and then mixed with 5 ml of methanol (solution B) before analysis of the simple and total flavonols. A volume of 2 ml taken from solution A was adjusted to 3 ml with 2 M HCl. The total (pro)anthocyanidins were measured by using an absorption spectrophotometer (UV-1201, Shimadzu) at 530 nm wavelength. The results were indicated as measurements of optical density because there were no standards. A volume of 100 µl of solution B was left to react for 20 min in 5 ml of a 1% AICl3 (w/w) methanol solution. Total flavonols were measured by absorption spectrophotometry at a wavelength of 435 nm (measurement of the formation of a colored complex in the presence of Al^{3+} ions). Quantitative results were expressed with reference to a standard set carried out by using quercetin.

Identification and Quantification of Simple Flavonols Extracts were analyzed by high-performance liquid chromatography with a UV detector (UVD170, Dionex) set at wavelength 370 nm and a Nucleosil 100 C18 column (4.6×250 mm) fitted with a stationary same-phase precolumn. The eluent composition was 1, water; 2, methanol; 3, tetrahydrofuran; and 4, acetic acid. The elution profile was from 57:20:17:6 (1/2/3/4) to 20:60:17:3 for 35 min with a constant flow rate of 1 ml min^{-1}. The injection volume was 20 µl. Identification of compounds was based on the comparison of their retention times and their UV spectra (250–400 nm) with those of commercial standards (Roth-Sochiel®). External calibration was performed with a mixture of four flavonoids: myricetin, quercetin, isorhamnetin, and kaempferol. These simple flavonols are the most used, according to the literature, for their response to variations in environmental conditions (Waterman and Mole 1994).

Statistical Analyses For each analysis, three replicates were made. The two-way ANOVA, a nonparametric test of median comparisons, Kruskall–Wallis, and Tukey tests were performed by using the "Statgraphics for Windows" software produced by Statistical Graphics®.

Results

Total (pro)Anthocyanidins and Total Flavonols Average concentrations of total (pro) anthocyanidins (Fig. 1a) are significantly lower in chilled (57% loss) and freeze-dried (71% loss) blades than in fresh and oven-dried ones (only 3% loss; Kruskal–Wallis, $P<0.05$) in *P. oceanica*. Average concentrations of total flavonols (Fig. 1b) are lower in freeze-dried samples (87% loss) compared to the other prehandling methods (39 and 2% losses for chilled and oven-dried samples, respectively; Kruskal–Wallis, $P<0.05$). No significant differences were found between fresh and oven-dried samples (Kruskal–Wallis, $P>0.05$) for (pro)anthocyanidins and total flavonols.

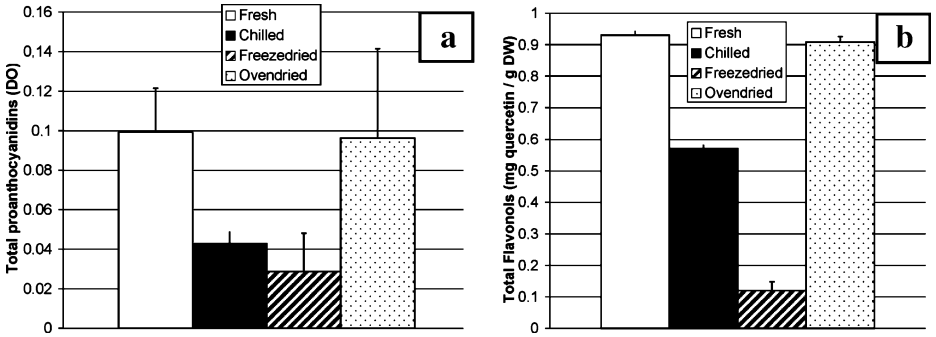


Fig. 1 Average concentrations of total flavonoids in fresh, chilled, freeze-dried, and oven-dried blades of *P. oceanica*. **a** Total proanthocyanidins [in optical density (*OD*) ± standard error]; **b** total flavonols (mg of quercetin g^{-1} of dry matter ± standard error)

Simple Flavonols All simple flavonols measured in *P. oceanica* samples were identified and classified according to their retention time: myricetin, quercetin, isorhamnetin, and kaempferol. Average concentrations of simple flavonols (Table 1) were lower in freeze-dried samples compared to the other prehandling methods (Kruskal–Wallis, $P<0.05$). No significant differences were found between fresh and oven-dried samples (Kruskal–Wallis, $P>0.05$) for any simple flavonols. Compared to fresh samples, for all simple flavonols, chilling led to a loss of 48%, freeze-drying to a loss of 95–98%, and oven drying to a loss of only 4–7% (two-way ANOVA, $P<0.05$).

Discussion

Concentrations of total and simple flavonols measured in *P. oceanica* are of the same order as those found in terrestrial species such as *Pinus halepensis* Mill. (Kaundun et al. 1998). Those identified in *P. oceanica* have been observed previously (Kaundun et al. 1998). Total and simple flavonols in *P. oceanica* are quantitatively influenced by the prehandling method, but their profiles are not. Concentrations of total and simple flavonols are considerably reduced by chilling or freeze-drying, but remain relatively unchanged by oven drying, compared to fresh samples.

Table 1 Mean concentrations in simple flavonols (μg g^{-1} of dry matter ± standard error) and rapport between fresh, chilled, freeze-dried, and oven-dried samples in the blades of *P. oceanica*

Simple Flavonols	Myricetin	Quercetin	Isorhamnetin	Kaempferol
Fresh	19.790±2.102 (c)	2.702±0.301 (c)	3.341±0.335 (c)	1.057±0.097 (c)
Chilled	10.292±0.989 (b)	1.405±0.137 (b)	1.737±0.198 (b)	0.55±0.057 (b)
Freeze-dried	0.382±0.031 (a)	0.066±0.006 (a)	0.160±0.011 (a)	0.046±0.004 (a)
Oven-dried	18.299±1.598 (c)	2.505±0.142 (c)	3.220±0.235 (c)	0.979±0.084 (c)
ANOVA results	$F=219.57$ $P<0.001$	$F=71.28$ $P<0.001$	$F=137.67$ $P<0.001$	$F=42.54$ $P<0.001$

Results of statistical analysis are also done for each flavonol type (F and p value). Tukey's tests with regard to treatment effects are also presented for each flavonol: values not different at $\alpha=5\%$ are denoted with the same letter.

Waterman and Mole (1994) explained that, after collection of plant material, enzymatic activity may quickly lead to irreversible changes in phenolic compounds, such as oxidation and subsequent polymerization or decomposition. After 48 hr of chilling, we saw the color of *P. oceanica* leaves becoming brownish, which indicated that chemical and enzymatic reactions were occurring (Julkunen-Tiitto and Tahvanainen 1989). This result emphasizes the importance of treating leaves immediately after sampling. The time spent in the fridge and/or icebox must be as short as possible and must always be the same from one sampling to another.

Freeze-drying drastically altered the quantity of measured flavonoids. There was a loss of 71% of total (pro)anthocyanidins, 87% of total flavonols, and 95% of all simple flavonols (myricetin, quercetin, isorhamnetin, and kaempferol). The impact of freeze-drying on phenolic compounds has been much debated. It seems to vary according to the chemical species, the part of the plant, and the plant considered. For example, Julkunen-Tiitto and Sorsa (2001) did not show any significant impact of freeze-drying on flavonoid glycosides in *Salix purpurea*, but Orians (1995) demonstrated a loss of 70% for some *Salix* sp. phenolic glycosides. The results observed by Steinberg (1986) seem to vary with the species, as freezing led to an 11% decrease in total phenolic compounds in *Sargassum globulariaefolium* but a 4–5% increase in *Sargassum* sp.

Chalker-Scott et al. (1989) observed a loss of phenolics of more of 40%, when Rhododendron leaves were frozen to −15°C and showed that losses are caused by temperatures lower than −5°C, the phenomenon being aggravated at lower temperatures. In our study, samples were frozen to −26°C, which may partly explain the significant losses. Freezing would break the membranes, simultaneously releasing compartmentalized phenolics and proteins into the cell (Chalker-Scott and Fuchigami 1989). Phenolics, once free in the cell, are likely to form hydrogen bonds with proteins to form insoluble complexes, by means of their hydroxyl groups (Chalker-Scott and Fuchigami 1989; Waterman and Mole 1994). Such modifications can lead to phenolic variation. Such changes may result in higher or lower concentrations, depending on the formula of the compound considered. Beyond the presence of hydroxyl groups, their particular disposition on the carbon skeleton and the molecular weight may also be important in how specific phenolic molecules interact with proteins (Waterman and Mole 1994).

Lindroth and Koss (1996) have explained the reduction in phenolic concentrations by hydrolytic degradation during thawing of frozen leaf tissue. This may occur during freeze-drying and not by freeze-drying *per se*, thus emphasizing the importance of using a temperature-regulated drying chamber (Orians 1995). This thawing phenomenon cannot alone be responsible for the decrease in flavonoid concentrations because the decreases are higher than those observed with chilling (when enzymatic decomposition is assumed to be maximal). The problem of thawing may be aggravated by the fact that, in parallel, the drying process slowly continues, leading to the concentration of enzymes and substrates, which would likely facilitate such reactions (Waterman and Mole 1994). Some phenolics might also be lost during freeze-drying under high-vacuum conditions by their being concentrated in the ice trap (Van Sumere et al. 1983 in Orians 1995).

Oven-drying seems to be an appropriate method for preserving flavonoids from *P. oceanica*, as the concentrations remain similar to those of fresh samples. Our results are in accordance with those reported by Julkunen-Tiitto and Tahvanainen (1989), who underline the importance of oven-drying intact leaves to minimize enzymatic decomposition that occurs when samples are stirred into a homogeneous mixture before drying. During oven drying, the disappearance of moisture is rapid in *P. oceanica*, even in entire leaves, because the species has very thin leaves (its growing cycle occurs entirely in submerged meadow, so

it is highly subject to desiccation). This is not systematically the case for most terrestrial species, which need to be cut into small pieces to accelerate the elimination of moisture. Rapid drying shortens the time that compounds are subject to enzyme action because the reactions require water (Julkunen-Tiitto and Tahvanainen 1989). The use of high temperatures (60 to 80°C) for drying, however, has been shown to reduce the amount of extractable total phenolics and (pro)anthocyanidins in the leaves of *Salix* sp. (Julkunen-Tiitto 1985 in Julkunen-Tiitto and Tahvanainen 1989).

Our results indicate that, when treating fresh samples immediately is not possible, oven drying for 48 hr at 40°C is preferable to freeze-drying for *P. oceanica*. Although there were significant differences among the different methods regarding flavonoid concentrations, none altered the flavonoid profile.

Acknowledgments This work was carried out and funded in the framework of the Interreg III European Programs. The authors thank C. Lafabrie and B. Mimault for participation in field missions.

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J Chem Ecol (2007) 33:1089–1103
DOI 10.1007/s10886-007-9286-1

Rice Allelopathy Induced by Methyl Jasmonate and Methyl Salicylate

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Received: 29 November 2006 / Revised: 5 March 2007 / Accepted: 14 March 2007 /
Published online: 6 April 2007
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Abstract Methyl jasmonate (MeJA) and methyl salicylate (MeSA) are important signaling molecules that induce plant defense against insect herbivores and microbial pathogens. We tested the hypothesis that allelopathy is an inducible defense mechanism, and that the JA and SA signaling pathways may activate allelochemicals release. Exogenous application of MeJA and MeSA to rice (*Oryza sativa* L.) enhanced rice allelopathic potential and led to accumulation of phenolics, an increase in enzymatic activities, and gene transcription of phenylalanine ammonia-lyase (*PAL*) and cinnamate 4-hydroxylase (*C4H*), two key enzymes in the phenylpropanoid pathway. Aqueous extracts of the leaves of rice IAC165, a putative allelopathic variety, treated with MeSA (5 mM) or MeJA (0.05 mM), showed increased inhibitory effects (25 and 21%, respectively) on root growth of barnyardgrass (*Echinochloa crus-galli* L.), and increased inhibitory effects (18 and 23%, respectively) on shoot growth. Aqueous extracts from leaves of Huajingxian 1 rice, a putative nonallelopathic variety treated with MeJA and MeSA, caused 63 and 24% inhibition of root growth in barnyardgrass seedlings. The root exudates of both IAC165 and Huajingxian 1 plants treated with MeJA and MeSA for 48 hr also showed significant increases in their inhibitory effects on root growth of barnyardgrass seedlings. At the four-leaf stage, levels of 3,4-hydroxybenzoic acid, vanillic acid, coumaric acid, and ferulic acid that accumulated in the

EH Graham Centre for Agricultural Innovation is a collaborative alliance between Charles Sturt University and the New South Wales Department of Primary Industries

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leaves were 5.3-, 31.3-, 2.2-, and 1.7-fold higher in response to MeJA exposure, and 3.3-, 13.1-, 2.0-, and 2.2-fold higher in response to MeSA. Treatments of MeSA and MeJA enhanced the PAL activity in the rice leaves up to 52.3 and 80.1%, respectively, whereas C4H activity was increased by 40.2 and 67%. Gene transcription of *PAL* and *C4H* in rice leaves significantly increased after the plants were subjected to treatment with MeJA and MeSA. These results suggest that allelopathy may be an active defense mechanism, and that plant signaling compounds are potentially valuable in its regulation.

Keywords Methyl salicylate · Methyl jasmonate · Rice · Allelopathy · Inducible · Phenolic acid · Phenylalanine ammonia-lyase · Cinnamate 4-hydroxylase

Abbreviations

MeSA	methyl salicylate
MeJA	methyl jasmonate
VA	vanillic acid
CA	caffeic acid
FA	ferulic acid
CMA	coumaric acid
HBA	3,4-hydroxybenzoic acid
PAL	phenylalanine ammonia-lyase
C4H	cinnamate 4-hydroxylase

Introduction

Allelopathy provides an alternative for weed management in sustainable agriculture. Rice (*Oryza sativa* L.) is one of the most important crops in the world, and its allelopathy has attracted great attention since Dilday et al. (1989) demonstrated that some varieties have allelopathic potential against one or more paddy weeds. Rice allelopathy has been extensively studied with respect to screening allelopathic rice germplasm (Dilday et al. 1994; Olofsdotter et al. 1995, 1999), the allelochemicals produced (Mattice et al. 1998; Kato-Noguchi 2004; Seal et al. 2004a, b), and genetic control (Ebana et al. 2001; Jensen et al. 2001; Zeng et al. 2003; He et al. 2004). More than 12,000 rice accessions have been evaluated in the United States for their allelopathic potential of weed control in paddy fields (Dilday et al. 1994, 1998). Selection of allelopathic rice germplasm has been conducted in many countries (Fujii 1992; Garrity et al. 1992; Dilday et al. 1994; Olofsdotter et al. 1995; Chung et al. 1997, 2000, 2001a, b; Chou 1999; Ahn and Chung 2000). Work by Seal et al. (2005) has shown the correlation between the allelopathic potential of rice, as measured in the laboratory, and weed inhibition in the field. Several putative allelochemicals have been identified, including simple phenolic acids such as *p*-hydroxybenzoic (Chou and Lin 1976), ferulic (Chung et al. 2000, 2001a), syringic, caffeic, sinapic, and *o*-coumaric acids (Olofsdotter et al. 1995). Recent studies have indicated that momilactone A and B may play an important role in rice allelopathy (Kato-Noguchi et al. 2002; Kato-Noguchi 2004; Chung et al. 2006). Kato-Noguchi and Ino (2003) found that momilactone B from rice seedlings could accumulate to levels high enough to inhibit growth of cress seedlings. 3-Isopropyl-5-acetoxycyclohexene-2-one-1, momilactone B, and 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone were also identified as putative allelochemicals in the rice accession PI312777 (Kong et al. 2004).

The genetic control of allelopathy in rice has been assessed. Jensen et al. (2001) identified four main-effect quantitative trait locus (QTL) genes located on three chromosomes (2, 3, and 8), which collectively explained 35% of the total phenotypic variation of the allelopathic activity in the population. Ebana et al. (2001) also identified QTL genes associated with the effect by using restriction fragment length polymorphism markers. One of the QTLs on chromosome 6 had the largest effect, explaining 16.1% of the phenotypic variation. He et al. (2005) employed proteomic methods to study the molecular mechanism of crop allelopathy and identified four proteins involved in the production of allelochemicals: peroxidase precursor, thioredoxin M-type, 3-hydroxy-3-methylglutaryl-coenzyme A reductase 3, and phenylalanine ammonia-lyase (PAL). The genes encoding these four allelopathy-related proteins are located on chromosomes 4, 7, 8, and 12.

Upon attack by herbivores and pathogens, plants may produce chemicals that combat their enemies. Chemical defense of plants is ubiquitous, often inducible, and activated through a complex network of signaling cascades involving jasmonate and salicylate pathways. Jasmonates and salicylates function as key signal molecules in plant defense (Kessler and Baldwin 2002) and modulate plant resistance to insects and pathogens (Creelman and Mullet 1997). Many defense-related genes require jasmonic acid (JA) and salicylic acid (SA) for activation (Reinbothe et al. 1994; Thomma et al. 1998; Turner et al. 2002). Exogenously applied JA induces the production of momilactone A, a major phytoalexin and possible allelochemicals in rice (Nojiri et al. 1996), and increases resistance of wild plants to insects in the field (Baldwin 1998). Poplar leaves increase phenolic acids when exogenously treated with methyl salicylate (MeSA) and methyl jasmonate (MeJA) (An et al. 2006). Sakuranetin, a flavonoid phytoalexin, is also induced by amino acid conjugates of JA (Shigeru et al. 1997). Two acyclic homoterpenes 4,8-dimethyl-1,3*E*,7-dimethylnonatriene (homoterpene I) and 4,8,12-trimethyl-1,3*E*,7*E*,11-tridecatetraene (homoterpene II), which are of sesquiterpenoid and diterpenoid origin, can be induced by 0.1 to10 mM of JA on leaves of *Phaseolus lunatus* and *Zea mays* (Jörn et al. 1994). Methyl salicylate treatment increased concentrations of *u*-tropine, and tropine in jimsonweed (*Datura stramonium*) seedlings (Fan 2005) and induced triterpenoid synthesis in both *Centella asiatica* and *Galphimia glauca* plantlets (Mangas et al. 2006). MeJA also induced indole glucosinolate biosynthesis in *Arabidopsis* (Brader et al. 2001) and oilseed rape (Loivamaki et al. 2004). Salicylic acid is a well-known inducer of plant systematically acquired resistance in plant–pathogen interactions, which results in resistance to pathogens and is characterized by the induction of defense-related gene expression and synthesis of phenylpropanoids, phytoalexins, and pathogenesis-related proteins (Métraux 2001; Durrant and Dong 2004; De Vos et al. 2005). Salicylic acid also induces the biosynthesis of secondary metabolites in plants (Taguchi et al. 2001).

Secondary compounds can enhance the survival of a plant, allowing greater reproduction in stressful environments. It is still unclear, however, whether the release of allelochemicals into the environment is passive or active. It is hypothesized that allelopathy is an actively inducible defense mechanism, and that the JA and SA signaling pathways may activate chemical release. In this study, we tested the above hypothesis by applying MeJA and MeSA to rice exogenously and by examining changes in the allelopathic potential of rice exudates and aqueous extracts, as well as in gene expression and in enzymatic activities of PAL, which catalyzes the first step in the biosynthesis of phenylpropanoids and cinnamate 4-hydroxylase (C4H). The latter catalyzes the conversion of cinnamate into 4-hydroxy-cinnamate, a key reaction in the phenylpropanoid pathway.

Methods and Materials

Plant and Chemical Materials

Two rice varieties were used: IAC165, a standard cultivar with known allelopathic potential (Kim et al. 2005) was provided by the International Rice Research Institute, and Huajingxian 1, a nonallelopathic cultivar (Bi 2004), was provided by Prof. Zhi-qiang Chen at the South China Agricultural University. Vanillic acid (VA), caffeic acid (CA), 3,4-hydroxybenzoic acid (HBA), ferulic acid (FA), coumaric acid (CMA), MeJA, and MeSA were purchased from Sigma (St. Louis, MO, USA). The concentrations of MeJA and MeSA used were 0.05 and 5 mM, respectively. These two were chosen because they are well-established signal molecules belonging to an integral part of a general signal transduction system in plant defense responses, and they are volatile signals whose potential phytotoxicity could be avoided (Preston et al. 2002). TRIzol reagent, AMV reverse transcriptase, *Taq* polymerase, and deoxynucleotide triphosphates were purchased from TaKaRa (Shuzo Co. Ltd., Shiga, Japan). MOPS and DEPC were purchased from AMRESCO (Solon, OH, USA). All solvents used were high-performance liquid chromatography (HPLC) grades.

Bioassays

Rice seeds were surface-sterilized with 1% NaClO for 30 min, rinsed with distilled water, and pregerminated in petri dishes for 3 d. Twenty germinated seeds were transplanted to a plastic pot (10×15 cm) and raised in a growth chamber kept at 24–26°C with 150 μMd m^{-2} sec^{-1} light and a photoperiod of 12-hr light/12-hr darkness. The pots were watered and fertilized with Hoagland nutrient solution every 2 d.

Rice seedlings at the two-, four-, and six-leaf stages were sprayed with 0.05 mM of MeJA or 5 mM of MeSA and kept in the growth chamber for 48 hr. Aqueous extracts were prepared by extracting 8 g of plant leaves and stems with 100 ml of distilled water for 24 hr. Extracts were filtered through filter paper and stored at 4°C until used.

Barnyardgrass (*Echinochloa crus-galli*) seeds were used to bioassay the aqueous extracts of rice leaves and stems, as described by Zeng et al. (2001). Root and shoot lengths of the *E. crus-galli* seedlings were determined 7 d after treatment.

Root Exudates

Pregerminated seeds of rice (10 seeds per beaker) and barnyardgrass (20 seeds per beaker) were planted in a 1,000-ml beaker with 300 ml of 1% agar culture media. The agar medium was divided into two equal compartments by using a membrane with a pore size of 35 μm. The membrane prevented root contact between the plants but allowed root exudates to pass through. Rice and barnyardgrass seedlings were grown in the growth chamber as described above. Leaves were painted with 0.05 mM of MeJA or 5 mM of MeSA three times with a brush pen—at the two-, four-, and six-leaf stages. The barnyardgrass seedlings and culture medium were wrapped in aluminum foil to prevent contact with signaling compounds as the compounds were applied. Seedlings were watered and fertilized with Hoagland nutrient

solution every 2 d. Root and shoot lengths of the *E. crus-galli* seedlings were measured 7 d after the rice plants received the final application of signaling compounds.

Chemical Analysis

Plants treated with MeJA or MeSA at the four-leaf stage were kept in the growth chamber for another 48 hr before sampling. One gram of rice leaves and stems was collected from the different treatments and extracted in 12.5 ml of distilled water for 24 hr. Water extracts were partitioned against an equal volume of ethyl acetate three times. The ethyl acetate extracts were combined and concentrated to dryness at 40°C under reduced pressure, then dissolved in 1.25 ml of methanol. Methanol extracts were passed through sterilized filter paper in a syringe. Phenolic compounds in the filtrate were analyzed by using an Agilent Technologies HP1100 series HPLC system equipped with an ODS reverse phase C18 column (250×4 mm, 5 μm) and diode array detector (G1315 B) monitoring the absorbance of the elution at 280 and 268 nm. The solvent system was 75% methanol and 25% water with pH adjusted to 2.6 with acetic acid. Five microliters of each extracted sample were injected, flow rate was 1.0 ml/min, and temperature was 30°C. Pure compounds were used as standards, and phenolic acids were identified by comparison of retention times and UV spectrum.

Enzymatic Activities

Rice plants were treated with signal compounds at the four-leaf stage for the C4H assay, and at the two-, four-, and six-leaf stages for the PAL assay. Before the enzymatic activity analysis, the plants were sprayed with MeJA (0.05 mM) or MeSA (5 mM) and kept in different growth chambers for another 48 hr. Phenylalanine ammonia-lyase activity was assayed based on the method described by Ramamoorthy et al. (2002) with slight modification. Root samples (1 g) were ground with liquid nitrogen and homogenized in 1 ml of ice-cold 0.1 M Tris–H_2SO_4 buffer, pH 8.8, containing 7 mM of 2-mercaptoethanol and 1 mM EDTA–Na, 7% glycerin. The homogenate was centrifuged (10,000×*g* for 15 min). The supernatant was used for enzyme analysis.

PAL activity was determined based on the rate of the conversion of L-phenylalanine to *trans*-cinnamic acid, as measured by absorbance at 290 nm. The absorbance (OD1) of 0.5 ml of enzyme extract, 2 ml of 50 mM Tris–H_2SO_4 buffer (pH 8.8), and 1 ml of 20 mM L-phenylalanine was measured before and after incubation in hot water for 15 min at 40°C. Enzyme activity was stopped after incubation with the addition of 6 M HCl. Phenylalanine ammonia-lyase activity was determined spectrophotometrically as described by Edwards and Kessmann (1992) and was expressed in units per gram per hour.

The C4H activity was assayed by using the method described by Lamb and Rubery 1975 with slight modification. Cinnamate 4-hydroxylase was extracted from fresh rice leaves (1 g), then homogenized in 2 ml of potassium phosphate buffer (200 mM, pH 7.5, containing 2 mM of 2-mercaptoethanol). After filtration and centrifugation (15 min at 10,000×*g*), the supernatant was diluted 20 times, and used for enzymatic analysis. The extract (0.2 ml) was added to 2 ml of reaction buffer (50 mM phosphate buffer containing 2 mM of 2-mercaptoethanol, 2 mM *trans*-cinnamic acid, and 0.5 mM NADPH), which was incubated for 1 hr at 37°C. Absorbance values were measured at 290 nm after the reaction

was stopped with 6 M of HCl and readjusted to pH 11 with 6 M of NaOH. The C4H activity was expressed in units per gram per hour.

Reverse Transcription Polymerase Chain Reaction

Rice plants at the four-leaf stage were sprayed with MeJA and MeSA and kept in growth chambers for 48 hr before sampling. The total RNA was extracted and isolated according to the method described by Kiefer et al. (2000) with slight modification. Fresh plant tissue (0.2 g) was ground with a mortar and pestle under liquid nitrogen, and the powdered tissue was transferred to a 2-ml Eppendorf tube, then 1,000 μl of TRIzol reagent was added and mixed. After incubation for 8–10 min on ice, 200 μl of chloroform were added and mixed. After 5 min of incubation at room temperature, the mixture was centrifuged for 15 min at 4°C and 12,000×*g*. The supernatant was transferred to a 1.5-ml Eppendorf tube, and 500 μl of isoamyl alcohol was added, followed by vortexing at room temperature for 10 min and centrifugation for another 10 min at 4°C and 13,000×*g*. The supernatant was discarded, and the pellet washed with 1 ml of 75% ethanol (v/v) and kept at −20°C until used.

The expression patterns of defense-related genes in rice leaves after treatment with signaling compounds were analyzed by using reverse transcription polymerase chain reaction (RT-PCR). The degenerate primers used for amplification of the putative genes are described in Table 1. Actin was used as a reference. Reverse transcription polymerase chain reactions were initiated with first strand cDNA synthesis at 42°C for 60 min. After denaturation of the RNA–cDNA hybrid at 94°C for 2 min, 40 PCR amplification cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) were run and followed by a final extension for 7 min. Two microliters of concentrated (×10) loading dye were added to each reaction, and 5 μl of each sample were run on a 1.2% agarose gel in 1×Tris–acetate–EDTA buffer.

Table 1 Specific primers for RT-PCR

Gene	Accession Number	Gene Function	Specific Primer
*Os*PAL	X16099	The first enzyme of the phenylpropanoid pathway catalyzing the deamination of phenylalanine to produce *trans*-cinnamic acid	F: 5′-CACAAGCTGAAGCACCACCC-3′ R: 5′-GAGTTCACGTCCTGGTTGTG-3′
*Os*C4H	OJ1342	A cytochrome P450 catalyzing the conversion of cinnamate into 4-hydroxy-cinnamate, a key reaction of the phenylpropanoid pathway	F: 5′-CTGGCACCGACGGTCATGTT-3′ R: 5′-CTGGATGGTGCTTGAGCTTG-3′
Actin	X15865	Constitutive actin gene and internal standard of RT-PCR	F: 5′-ACTGTCCCCATCTATGAAGGA-3′ R: 5′-CTGCTGGAATGTGCTGAGAGA-3′

Actin, internal standard of RT-PCR

C4H=Cinnamate 4-hydroxylase, PAL=phenylalanine ammonia-lyase

Statistical Analyses

All data were evaluated by one-way analysis of variance with treatment differences among means being tested at $P=0.05$ with Duncan's multiple range test. The root and shoot growth results as well as the content of phenolic acids and the enzymatic activities of PAL and C4H were means from three replicates.

Results

Aqueous Extract

The aqueous extracts of the stems and leaves of rice IAC165, a putative allelopathic variety, had much stronger phytotoxic effects on the root and shoot growth of barnyardgrass after being treated with MeSA (5 mM) or MeJA (0.05 mM) than the control, i.e., aqueous extracts from rice not receiving treatment with signaling compounds (Table 2). Aqueous extracts of the stems increased inhibition of the root growth in barnyardgrass by 24 and 32% and shoot growth by 38 and 27%, respectively. Aqueous extracts of the leaves increased inhibition of the root growth by 25 and 21% and the shoot growth by 18 and 23% (Table 2).

After treatment with signaling compounds, aqueous extracts of both leaves and stems of rice Huajingxian 1, a putative nonallelopathic variety, also showed stronger inhibition of the root growth in barnyardgrass compared with aqueous extracts from plants not receiving treatment (Table 2). Aqueous extracts from stems of Huajingxian 1 plants treated with MeJA (0.05 mM) or MeSA (5 mM) for 48 hr showed 63 and 36% higher inhibition of root growth compared with the control. Similarly, the aqueous extracts of leaves from Huajingxian 1 plants exposed to MeJA or MeSA also displayed higher inhibition of root growth by 63 and 24% compared with the control (Table 2), but the shoot length of barnyardgrass seedlings did not differ from the controls (Table 2). Bioassay results showed that both the allelopathic and nonallelopathic rice varieties enhanced their phytotoxicity against barnyardgrass after being treated with the signaling compounds MeSA and MeJA.

Table 2 Effects of the aqueous extracts from Huajingxian 1 and IAC165 rice treated with MeJA (0.05 mM) or MeSA (5 mM) on root length and shoot length of barnyardgrass

Extraction Organ	Treatments	Huajingxian 1		IAC165	
		Root Length (cm)	Shoot Length (cm)	Root Length (cm)	Shoot Length (cm)
Stems	Control	7.0±0.49a	6.0±0.18a	6.3±0.27a	6.0±0.49a
	MeJA	2.6±0.21c	5.9±0.42a	4.2±0.47b	4.4±0.42b
	MeSA	4.5±0.51b	5.6±0.23a	4.8±0.4b	3.7±0.39b
Leaves	Control	4.8±0.37a	5.5±0.25a	6.3±0.32a	5.6±0.34a
	MeJA	1.8±0.37c	5.2±0.25a	5.0±0.35b	4.3±0.28b
	MeSA	3.6±0.14b	6.0±0.31a	4.7±0.49b	4.5±0.32b

Rice plants were treated externally at the four-leaf stage for 48 hr. Values are mean±standard error. Significant difference ($P<0.05$) among treatments in each group are indicated by different letters.

Table 3 Effect of the root exudates of Huajingxian 1 and IAC165 rice treated with MeJA (0.05 mM) or MeSA (5 mM) on root length and shoot length of barnyardgrass grown in agar

Rice Cultivars	Treatments	Root Length (cm)	Shoot Length (cm)
Huajingxian 1	Control	10.9±0.44a	22.3±0.55a
	MeJA	8.4±0.44b	21.4±0.76a
	MeSA	8.8±0.36b	21.2±0.77a
IAC165	Control	10.4±0.74a	20.6±0.54a
	MeJA	8.6±0.56b	20.9±0.80a
	MeSA	8.4±0.55b	18.8±1.33a

Rice plants were treated externally at the four-leaf stage for 48 hr. Values are mean±standard error. Significant difference ($P<0.05$) among treatments in each group are indicated by different letters.

Root Exudates

The root exudates of rice plants treated with MeJA (0.05 mM) or MeSA (5 mM) for 48 hr significantly increased their inhibitory effects on root growth of barnyardgrass seedlings compared with the control, i.e., the root exudates of plants not treated with a signaling compound (Table 3). Application of MeJA increased inhibitory effects of Huajingxian 1 and IAC165 by 24 and 17%, respectively, and MeSA application increased it by 20 and 19%, respectively. Thus, application of signaling compounds increased the toxicity and allelopathic potential of root exudates for both allelopathic and nonallelopathic rice plants.

Phenolic Acids

Phenolic acids in the leaves and stems of rice IAC165 were analyzed with HPLC after plants were treated with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the four-leaf stage. An accumulation of HBA, VA, CMA, and FA was observed in the treated plants (Fig. 1). Coumaric acid and FA accumulated in the stems (Fig. 2), whereas HBA, VA, CMA, and FA in the leaves accumulated to levels of 5.3-, 31.3-, 2.2-, and 1.7-fold higher after MeJA treatment, and accumulated to levels of 3.3-, 13.1-, 2.0-, and 2.2-fold higher in response to MeSA (Fig. 1).

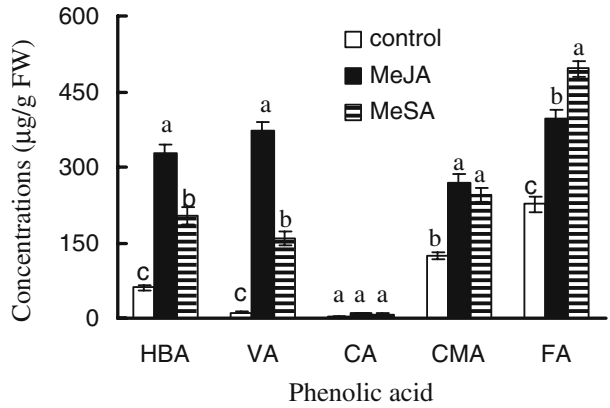


Fig. 1 Concentration of phenolic acids in aqueous leaf extracts of rice IAC165 after treatment with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the four-leaf stage. Values are means±SE from three replicates. Significant differences ($P<0.05$) among treatments in each group are indicated by different letters above bars. VA=Vanillic acid, CA=caffeic acid, FA=ferulic acid, CMA=coumaric acid; HBA=3, 4-hydroxybenzoic acid

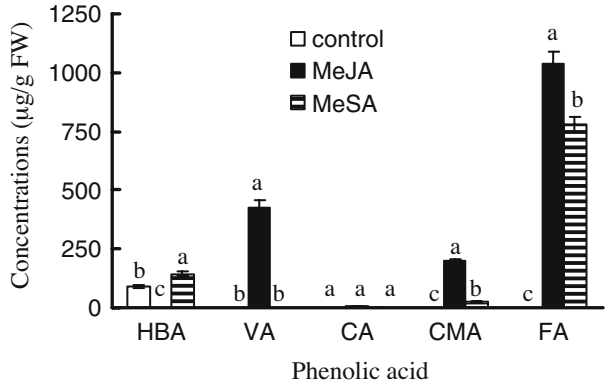


Fig. 2 Concentrations of phenolic acids in aqueous stem extracts of rice IAC165 after treatment with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the four-leaf stage. Values are means±SE from three replicates. Significant differences ($P<0.05$) among treatments in each group are indicated by different letters above bars. VA=Vanillic acid, CA=caffeic acid, FA=ferulic acid, CMA=coumaric acid; HBA=3, 4-hydroxybenzoic acid

Vanillic acid, CMA, and FA were not detected in the untreated rice stems, whereas MeJA induced high accumulation of VA, CMA, and FA in the stems of treated plants, and MeSA induced accumulation of CMA and FA in the stems (Fig. 2).

Phenylalanine Ammonia-lyase and Cinnamate 4-Hydroxylase Activity

Phenylalanine ammonia-lyase catalyzed the first step in the biosynthesis of phenylpropanoids. The activities of PAL in the leaves treated with MeSA (5 mM) or MeJA (0.05 mM) significantly increased compared with the control. Phenylalanine ammonia-lyase activities in the leaves treated with MeSA or MeJA at the two-leaf stage increased by 56 and 32%, respectively, compared with the control. At the four-leaf stage, MeSA and MeJA enhanced PAL activity by to 52 and 80%. Phenylalanine ammonia-lyase activity increased by 62.5 and 62.7%, respectively, when leaves were treated with MeSA or MeJA at the six-leaf stage (Fig. 3).

Forty-eight hours after treatment with MeSA or MeJA at the four-leaf stage, C4H activity in the rice IAC165 leaves increased by 40 and 67%, respectively, compared to leaves without a signaling treatment (Fig. 4).

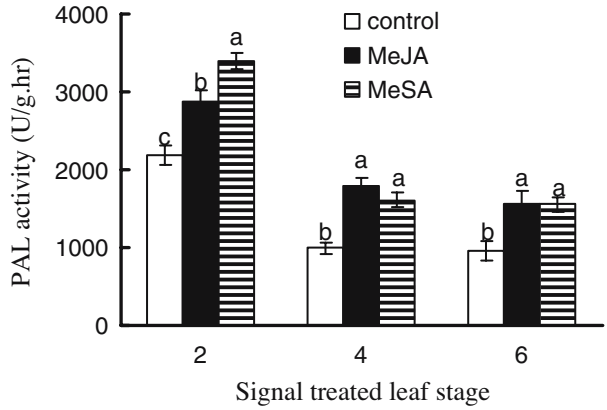


Fig. 3 Enzymatic activity of PAL in the leaves of rice IAC165 after treatment with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the two-, four-, and six-leaf stages. Values are means±SE from three replicates. Significant differences ($P<0.05$) among treatments in each group are indicated by different letters above the bars

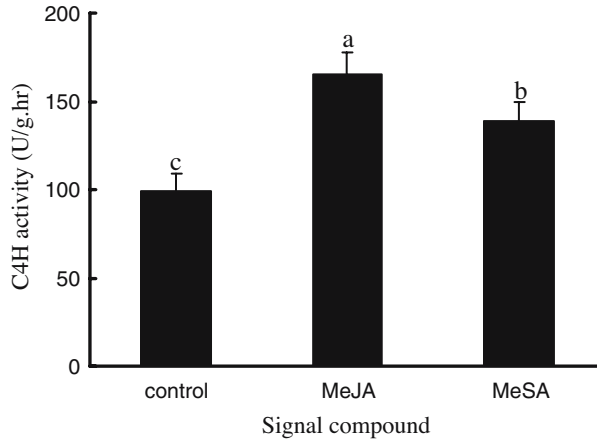


Fig. 4 Enzymatic activity of C4H in the leaves of the IAC165 after treatment with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the four-leaf stages. Values are means±SE from three replicates. Significant differences ($P<0.05$) among treatments are indicated by different letters above bars

Induction of Phenylalanine Ammonia-lyase and Cinnamate 4-Hydroxylase Transcripts

To determine whether MeJA and MeSA enhance the production of phenolic acids by inducing transcription of the genes encoding PAL and C4H, enzymes responsible for biosynthesis of phenylpropanoids, the expression patterns of PAL and C4H genes, were analyzed in the leaves of rice IAC165 after being treated with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the four-leaf stage. Both MeJA and MeSA induced accumulation of PAL and C4H transcripts over basal levels present in the control leaves (Fig. 5).

Discussion

Plants are frequently subjected to insect herbivory attack, pathogen infection, and competition from surrounding plants. In response, plants have evolved a diversity of chemicals that defend against herbivores and pathogens, and produce allelopathic effects on their neighboring competitors. Plant chemical defense against herbivores and pathogens are inducible and regulated by both the JA and SA signaling pathways. Exogenous addition of JA increases the resistance of wild plants to insects in the field (Baldwin 1998). Whereas many studies have shown that signal compounds can induce such defense against insect herbivores and microbial pathogens, rarely have experiments been conducted to demonstrate that signaling pathways are involved in allelopathy.

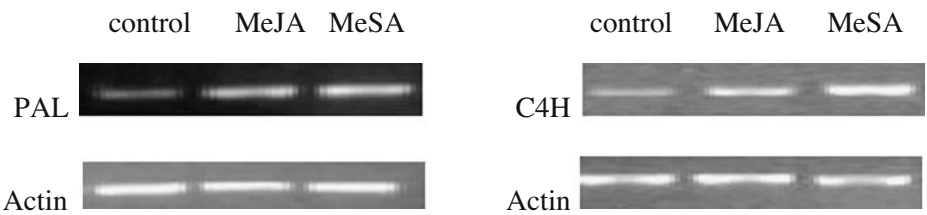


Fig. 5 Gene expression of *PAL* and *C4H* in the leaves of rice IAC165 in response to treatment with MeSA (5 mM) or MeJA (0.05 mM) for 48 hr at the four-leaf stage. Actin was used as internal standard of RT-PCR

This study found that rice allelochemicals could be induced by exogenous application of the signaling compounds MeJA and MeSA. The allelopathic potential of the root exudates of rice thus increased after treatment with MeJA and MeSA (Table 3). Aqueous extracts of both leaves and stems of MeSA- and MeJA-treated Huajingxian 1 and IAC165 rice cultivars showed enhanced inhibitory effects on barnyardgrass root growth (Table 2).

High-performance liquid chromatography analysis showed that phenolic acids VA, CA, CMA, FA, and HBA accumulated in rice leaves after being treated with MeJA and MeSA for 48 hr (Fig. 1). Caffeic acid, CMA, and FA were detected in the stems of rice plants treated with MeJA and MeSA, but not in the stems of untreated control plants (Fig. 2). Vanillic acid was also detected in the stem after treatment with MeJA. This increase in phenolic acids after application of signaling compounds may be a factor in the corresponding increase in the phytotoxicity of root exudates and aqueous extracts of induced rice plants. Similar changes in the production of several phenolic acids have been observed in poplar plants treated with MeJA (An et al. 2006).

Phenolics have been well documented in rice allelopathy (Chung et al. 2000, 2001a; Olofsdotter et al. 1995; Seal et al. 2004b). Greater quantities of *trans*-ferulic acid, *p*-hydroxybenzoic acid, and caffeic acid were detected in the exudates of allelopathic cultivars (Seal et al. 2004b). Five phenolics, caffeic, *p*-hydroxybenzoic, vanillic, syringic, and *p*-coumaric acids, that were found in rice exudates have been correlated with the observed allelopathic effects of rice on arrowhead (*Sagittaria montevidensis*) root growth (Seal et al. 2004b). Methyl jasmonate induced multiple biosynthetic pathways that included the shikimic acid pathway (producing MeSA), the octadecanoid pathway (producing *cis*-jasmone), as well as the mevalonate-dependent and mevalonate-independent terpenoid pathways (producing mono- and sesquiterpenes) (Gundlach et al. 1992; Thaler et al. 1996; Martin et al. 2002). The induction of these pathways has been demonstrated in agricultural plants, such as tomato (*Lycopersicon esculentum*), lima bean (*P. lunatus*), and corn (*Z. mays*) (Thaler et al. 1996; Dicke et al. 1999; Schmelz et al. 2003). Martin et al. (2003) recently demonstrated that application of MeJA induced the activity of both constitutive and novel terpene syntheses in Norway spruce (*Picea abies*).

Exogenous application of MeJA (0.05 mM) or MeSA (5 mM) increased PAL activity in rice at all three ages (two-, four-, and six-leaf stages) tested (Fig. 3), and also increased C4H activity in leaves at the four-leaf stage (Fig. 4). The genes coding for PAL and C4H that are constituents in the phenylpropanoid biosynthetic pathways leading to phenolic acid production were induced by exogenous application of MeJA and MeSA. Reverse transcriptase polymerase chain reaction showed that PAL and C4H mRNA level in the induced groups was greater than that in untreated plants (Fig. 5).

These results suggest that signaling compounds upregulate gene transcription of PAL and C4H, resulting in increased biosynthetic activities and accumulation of phenolic acids, which likely lead to enhanced allelopathic potential. The findings are ecologically significant in a sense that JA and SA signaling pathways may control the production and release of phytotoxins. Thus, plants may respond to competition or stress by activating signaling systems that release chemicals that interfere with a neighbor's growth. Wang et al. (2005) reported that rice plants showed increased allelopathic potential when in competition with barnyardgrass, flatsedge (*Cyperus difformis*), and ducksalad (*Monochoria vaginalis*). *Bidens pilosa* plants showed increased phytotoxicity in seasons of drought (Zeng and Luo 1995). Sorgoleone, the major phytotoxin in the root exudates of sorghum (*Sorghum bicolor*), was released in greater quantities in the presence of a crude extract of velvetleaf (*Abutilon theophrasti*) root, suggesting that seedling may increase their phytotoxicity and thus their allelopathic potential in the presence of other plants (Dayan 2006).

These findings also suggest that plant chemical defenses against herbivores, microbial pathogens, and other plants may share a common mechanism in which signal transduction plays a vital role. Cross resistance may occur. *Centaurea maculosa*, one of the most destructive invasive plants in North America, exuded higher amounts of (±)-catechin, a compound known to have inhibitory effects on native plants, when attacked by larvae of two different root boring biocontrol insects and a parasitic fungus (Thelen et al. 2005). Plant signals are potentially valuable for the regulation of allelopathy in competition with other plants. Understanding the signaling pathways involved will provide insight into plant chemical defense and help in the optimization of chemically based pest management programs.

Although recent experiments shed doubt on the importance of phenolic acids in rice allelopathy (Kato-Noguchi 2005), the correlation observed between the increase in rice inhibitory effects and the elevated production of phenolic acids in response to MeJA and MeSA exposure suggests the complexity of this issue. Plants produce an array of chemicals with different bioactivities, and a single compound is rarely responsible for a complicated biological process (Inderjit et al. 2006). More in-depth studies are required to determine the collective roles of phenolic acids and other putative allelochemicals in the overall allelopathic phenomenon of rice.

Acknowledgments This research was supported by the National 973 project of China (2006CB100200), Natural Science Foundation of China (30370246, 30670331), and Natural Science Foundation of Guangdong Province (group project 039254, 04105977), Guangdong Science and Technology Plan Program (2004B20501010, 2002C20506) and the Program for New Century Excellent Talents in University (NCET-04-0830) to R. S. Z. We thank three anonymous reviewers for helpful comments. We also thank Reed Johnson at University of Illinois at Urbana-Champaign for language revision.

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J Chem Ecol (2007) 33:1105–1113
DOI 10.1007/s10886-007-9279-0

RAPID COMMUNICATION

Chemical Composition of Inks of Diverse Marine Molluscs Suggests Convergent Chemical Defenses

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Received: 21 August 2006 / Revised: 9 January 2007 / Accepted: 2 March 2007 /
Published online: 29 March 2007
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Abstract Some marine molluscs, notably sea hares, cuttlefish, squid, and octopus, release ink when attacked by predators. The sea hare *Aplysia californica* releases secretions from the ink gland and opaline gland that protect individuals from injury or death from predatory spiny lobsters through a combination of mechanisms that include chemical deterrence, sensory disruption, and phagomimicry. The latter two mechanisms are facilitated by millimolar concentrations of free amino acids (FAA) in sea hare ink and opaline, which stimulate the chemosensory systems of predators, ultimately leading to escape by sea hares. We hypothesize that other inking molluscs use sensory disruption and/or phagomimicry as a chemical defense. To investigate this, we examined concentrations of 21 FAA and ammonium in the defensive secretions of nine species of inking molluscs: three sea hares (*Aplysia californica*, *Aplysia dactylomela*, *Aplysia juliana*) and six cephalopods (cuttlefish: *Sepia officinalis*; squid: *Loligo pealei*, *Lolliguncula brevis*, *Dosidicus gigas*; octopus: *Octopus vulgaris*, *Octopus bimaculoides*). We found millimolar levels of total FAA and ammonium in these secretions, and the FAA in highest concentration were taurine, aspartic acid, glutamic acid, alanine, and lysine. Crustaceans and fish, which are major predators of these molluscs, have specific receptor systems for these FAA. Our chemical analysis supports the hypothesis that inking molluscs have the potential to use sensory disruption and/or phagomimicry as a chemical defense.

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Keywords Amino acid · Ammonium · Anaspidea · *Aplysia* · Aplysioidea · Cephalopoda · Chemoreception · Cuttlefish · Gastropoda · Ink · Octopus · Opisthobranchia · Phagomimicry · Sea hare · Sensory disruption · Squid · Taurine

Introduction

Animals use a variety of defenses against predators, among them chemical defenses. Marine molluscs, including sea hares, squid, octopus, and cuttlefish, have a striking defensive behavior—releasing ink when attacked. Unlike cephalopods, sea hares often simultaneously release secretions from two glands, the ink and opaline glands (Johnson and Willows 1999). As the cephalopod ink sac is not homologous with the sea hare secretory glands, any functional similarities in the secretions would be the result of convergent evolution.

Sea hare secretions function against predators in at least three ways: chemical deterrence, sensory disruption, and phagomimicry. Chemical deterrents, which are typically unpalatable or toxic, inhibit feeding of predators on sea hares (Johnson and Willows 1999; Kicklighter and Derby 2006). Sensory disruption results from secretions massively stimulating the predator's chemosensory systems, preventing normal function, which leads to confusion and cessation of attack by the predator (Kicklighter et al. 2005). Phagomimicry results from secretions stimulating the predator's sensory pathways involved in feeding, thus causing the predator to attend to the secretions as if they were food and thus affording the sea hare an opportunity to escape (Johnson 2002; Kicklighter et al. 2005). The active compounds involved in sensory disruption and phagomimicry include free amino acids (FAA) and ammonium, which are extraordinarily concentrated in ink and opaline of the sea hare *Aplysia californica* and are powerful stimulants of the chemosensory neurons and feeding behavior of the spiny lobster *Panulirus interruptus*, a sympatric predator (Kicklighter et al. 2005).

Cephalopod inks function as anti-predatory visual stimuli, either as "smoke screens" or distracting decoys (Caldwell 2005). Some have proposed that cephalopod inks may contain compounds that disrupt a predator's chemical senses, but evidence is fragmentary or anecdotal (Caldwell 2005).

Our hypothesis is that inks from diverse cephalopods serve not only as visual defenses but also as chemical defenses against a diversity of predators, through sensory disruption, phagomimicry, or other mechanisms similar to those elucidated for *A. californica* against *P. interruptus*. As a first test, we analyzed FAA composition of the inks of three species of sea hares and six species of cephalopods, and compared these with FAA in typical food of predators of molluscs.

Methods and Materials

Aplysia californica individuals were field-collected in California (Marinus Scientific Inc., Garden Grove, CA, USA). Secretions from the ink and opaline glands were collected and pooled from one set of 15 individuals, and from a second set of 10 animals. Hemolymph from *A. californica* was collected from five animals. Mucus was collected from the skin of five animals. Ink and opaline glands were also collected from five *A. californica* raised in the NIH/University of Miami National Resource for *Aplysia* Facility and fed the red alga

Gracilaria ferox. The ink and opaline glands were taken from 10 *Aplysia dactylomela* collected in the Florida Keys. The opaline glands were taken from eight *Aplysia juliana* collected in waters off New Zealand.

Ink collected from the ink sacs of the following squid species were analyzed: three *Lolliguncula brevis* (collected near Galveston, TX, USA); three *Loligo pealei* (collected near Woods Hole, MA, USA); and one *Dosidicus gigas* (collected in California). In addition, ink was collected from the ink sacs of two *Octopus bimaculoides* (National Resource Center for Cephalopods) and one *Octopus vulgaris* (collected in waters near St. Augustine, FL, USA). Ink from three cuttlefish *Sepia officinalis* (collected near Galveston, TX) was also analyzed.

All species were collected in the field, except for the one group of *A. californica* raised in the NIH *Aplysia* facility, and *S. officinalis* cultured at the National Resource Center for Cephalopods. All animals, except for *L. brevis*, were adults and were alive until immediately before collection of the tissue or secretions. Sea hares were cooled until immobilized and then injected with isotonic magnesium chloride. Cephalopods were anesthetized by cooling on ice. Animals were dissected to remove the tissues of interest, which were frozen until used. Frozen samples were thawed, and secretions were collected. Ink and opaline were collected as previously described (Kicklighter et al. 2005; Kicklighter and Derby 2006). Hemolymph was collected from sea hares by first de-inking the animals to avoid contamination of hemolymph with ink and opaline, cold anesthetizing them, washing an area of skin between the rhinophores with 70% ethanol, making a small incision in that region, and collecting the released hemolymph into a sterile tube. Mucus was collected from sea hares by gently scraping the surface of the skin with a blunt probe. Secretions or tissue collected from multiple animals were pooled. Frozen samples were lyophilized and stored until used, at which time they were diluted in deionized water. Proteins were removed from the samples by dialysis. Concentrations of FAA and ammonium in the samples were analyzed by using a Beckman Model 6300/7300 amino acid analyzer by Scientific Research Consortium, Inc. (http://www.aminoacids.com). This system uses an ion exchange column, post-column ninhydrin reaction at 131°C, followed by absorbance measurements at 440 and 570 nm. (*S*)-2-Aminoethyl-1-cysteine and glucosaminic acid were used as internal standards. Data were acquired and managed with Beckman System Gold 8.10 chromatography software.

Multidimensional scaling (MDS) was used to examine the degree of similarity in the FAA composition of molluscan secretions and fluids, and food tissues. Our analysis used the proportional (%) compositions of the 20 secretions in Table 1 plus an equidose mixture (E). E, which contains the same 21 FAA as the mixtures in Table 1 but has all FAA at the same relative concentration (4.76), was included in this analysis for comparison. MDS was performed by using SPSS-X software and Euclidean values as a distance measure. The dimensionality of an acceptable MDS solution is the minimum that produces a stress value <0.10 (Kruskal and Wish 1978). In our analysis, a 3-dimensional solution produced a stress value of 0.072 and was thus accepted.

Results and Discussion

Sensory disruption requires that a secretion has the type and amount of FAA sufficient to strongly stimulate the sensory pathways of predators, although not necessarily mimicking the composition of food. Phagomimicry requires that a secretion's absolute and relative

Table 1 Chemical composition of molluscan secretions and other tissues[a]

Chemical in μM[a]	*A. californica* Wild opaline #1 So1	*A. californica* Wild opaline #2 So2	*A. californica* Wild ink #1 Si1	*A. californica* Wild ink #2 Si2	*A. californica* Mariculture ink Si3	*A. californica* Hemolymph Sf1	*A. californica* Mucus Sf2
Taurine	231,200 (74.5)	221,000 (54.7)	7,830 (52.9)	5,994 (49.3)	1,743 (86.0)	194 (27.1)	90 (15.9)
L-Aspartic acid	2,512 (0.8)	11,222 (2.8)	2,231 (15.1)	1,636 (13.5)	62 (3.1)	8 (1.1)	84 (14.9)
L-Threonine	236 (0.1)	182 (0)	193 (1.3)	173 (1.4)	3 (0.2)	10 (1.4)	6 (1.0)
L-Serine	68 (0)	270 (0.1)	214 (1.4)	123 (1.0)	7 (0.4)	13 (1.8)	26 (4.6)
L-Asparagine	41 (0)	0 (0)	51 (0.3)	0 (0)	1 (0.1)	6 (0.8)	4 (0.7)
L-Glutamic acid	1,616 (0.5)	3,970 (1.0)	1,166 (7.9)	1,394 (11.5)	27 (1.3)	4 (0.6)	14 (2.5)
L-Glutamine	9 (0)	0 (0)	121 (0.8)	72 (0.6)	6 (0.3)	17 (2.4)	8 (1.4)
L-Proline	7 (0)	122 (0)	131 (0.9)	124 (1.0)	8 (0.4)	0 (0)	2 (0.4)
L-Glycine	791 (0.3)	3,390 (0.8)	181 (1.2)	170 (1.4)	62 (3.1)	31 (4.3)	164 (29.1)
L-Alanine	339 (0.1)	416 (0.1)	1,024 (6.9)	879 (7.2)	21 (1.0)	64 (9.0)	29 (5.1)
L-Valine	56 (0)	328 (0.1)	301 (2.0)	189 (1.6)	15 (0.7)	10 (1.4)	12 (2.1)
L-Cystine	16 (0)	62 (0)	84 (0.6)	54 (0.4)	2 (0.1)	3 (0.4)	9 (1.6)
L-Methionine	35 (0)	102 (0)	122 (0.8)	29 (0.2)	6 (0.3)	4 (0.6)	1 (0.1)
L-Isoleucine	96 (0)	0 (0)	135 (0.9)	151 (1.2)	1 (0)	3 (0.4)	1 (0.1)
L-Leucine	10 (0)	160 (0)	327 (2.2)	144 (1.2)	4 (0.2)	6 (0.8)	2 (0.4)
L-Tyrosine	14 (0)	74 (0)	297 (2.0)	142 (1.2)	13 (0.6)	3 (0.4)	84 (15.0)
L-Phenylalanine	648 (0.2)	0 (0)	130 (0.9)	39 (0.3)	1 (0.1)	8 (1.1)	1 (0.1)
L-Tryptophan	0 (0)	0 (0)	5 (0)	0 (0)	0	1 (0.1)	0 (0)
L-Lysine	65,190 (21.0)	145,320 (35.9)	0 (0)	2 (0)	1 (0.1)	20 (2.8)	9 (1.6)
L-Histidine	7,185 (2.3)	17,237 (4.3)	255 (1.7)	844 (6.9)	20 (1.0)	14 (2.0)	15 (2.7)
L-Arginine	340 (0.1)	512 (0.1)	0 (0)	0 (0)	23 (1.1)	296 (41.4)	2 (0.4)
Total (TFAAs) μM	310409	404366	14798	12159	2026	715	563
Total (TFAAs) mM	310.4	404.4	14.8	12.2	2.0	0.7	0.6
Ammonium (μM)	6,810	5,336	24,360	32,305	15,180	272	2,852

concentrations of FAA adequately mimic that of the predators' food. Both mechanisms are supported by our chemical data.

The concentrations of FAA in ink and opaline of wild-caught sea hares are in the high millimolar range (Table 1). Opalines of wild-caught *A. californica* and *A. dactylomela* have the highest concentrations, 310–448 mM total FAA (TFAA), and opaline of *A. juliana* has 3.2 mM TFAA. Inks of wild-caught *A. californica* and *A. dactylomela* have 12–15 mM TFAA. Ink of maricultured *A. californica* contains about 15% of the TFAA in wild-caught animals, but the relative amounts of FAA acids are similar. Ink or opaline from two groups

Table 1 (continued)

A. dactylomela Opaline	*A. dactylomela* Ink	*A. juliana* Opaline	*Sepia officinalis* Ink	*Loligo pealei* Ink	*Lolliguncula brevis* Ink	*Dosidicus gigas* Ink
So3	Si4	So4	C1	C2	C3	C4
215,350 (48.1)	4,900 (41.3)	1,384 (43.2)	2,088 (82.1)	1,264 (75.5)	274 (60.2)	1,773 (83.9)
21,300 (4.8)	1,388 (11.7)	52 (1.6)	32 (1.3)	30 (1.8)	15 (3.3)	14 (0.7)
186 (0)	177 (1.5)	31 (1.0)	17 (0.7)	15 (0.9)	10 (2.2)	8 (0.4)
189 (0)	138 (1.2)	38 (1.2)	20 (0.8)	15 (0.9)	9 (2.0)	11 (0.5)
0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
83,800 (18.7)	1,780 (15.0)	469 (14.6)	135 (5.3)	66 (3.9)	26 (5.6)	71 (3.4)
0 (0)	104 (0.9)	0 (0)	2 (0.1)	4 (0.3)	1 (0.2)	6 (0.3)
170 (0)	159 (1.3)	28 (0.9)	49 (1.9)	107 (6.4)	8 (1.8)	37 (1.8)
2,554 (0.6)	735 (6.2)	47 (1.5)	6 (0.2)	8 (0.5)	4 (0.8)	26 (1.2)
2,063 (0.5)	859 (7.2)	139 (4.3)	134 (5.3)	88 (5.3)	59 (12.9)	43 (2.0)
312 (0.1)	273 (2.3)	33 (1.0)	30 (1.2)	21 (1.2)	14 (3.0)	11 (0.5)
14 (0)	250 (2.1)	2 (0.1)	0 (0)	5 (0.3)	0 (0)	0 (0)
17 (0)	323 (2.7)	15 (0.5)	0 (0)	1 (0)	0.4 (0.1)	14 (0.7)
118 (0)	108 (0.9)	20 (0.6)	10 (0.4)	15 (0.9)	9 (1.9)	7 (0.3)
188 (0)	195 (1.6)	29 (0.9)	17 (0.7)	29 (1.7)	20 (4.4)	12 (0.6)
71 (0)	0 (0)	14 (0.4)	0 (0)	0 (0)	0 (0)	0.7 (0)
75 (0)	343 (2.9)	13 (0.4)	0 (0)	3 (0.2)	4 (1.0)	2 (0.1)
496 (0.1)	18 (0.2)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
118,850 (26.6)	110 (0.9)	832 (26.0)	2 (0.1)	1 (0.1)	0.8 (0.2)	6 (0.3)
1672 (0.4)	0 (0)	10 (0.3)	0 (0)	0.0	0.6 (0.1)	11 (0.5)
212 (0)	0 (0)	48 (1.5)	0 (0)	2 (0.1)	1 (0.3)	61 (2.9)
447637	11,860	3203	2542	1675	455	2114
447.6	11.9	3.2	2.5	1.7	0.46	2.1
36,400	12,000	185	4,595	310	216	423

of 10–15 wild/caught *A. californica* collected at different times and places had similar TFAA, demonstrating a relative constancy at the population level.

Taurine, a sulfonic amino acid, is the major component in most secretions, constituting 41–86% of the TFAA. A few other FAA are in relatively high concentration, notably aspartic acid, glutamic acid, alanine, and lysine. Ammonium levels in *A. californica* and *A. dactylomela* ink or opaline are also high, 5–36 mM.

Cephalopod ink has lower millimolar levels of TFAA: 132 mM in *Octopus vulgaris*, 14.4 mM in *O. bimaculoides*, 2.5 mM in *Sepia officinalis*, 2.1 mM in *Dosidicus gigas*, 1.7 mM in *Loligo pealei*, and 0.5 mM in *Lolliguncula brevis* (Table 1). Taurine is typically

Table 1 (continued)

Octopus bimaculoides Ink C5	*Octopus vulgaris* Ink C6	*Callinectes sapidus* Muscle[b] T1	*Penaeus duorarum* Muscle[b] T2	*Crassostrea virginica* Body[b] T3	*Mugil cephalus* Muscle[b] T4
1,171 (8.1)	62,448 (47.1)	11,300 (3.9)	33,000 (17.5)	33,100 (62.7)	11,400 (39.1)
802 (5.6)	2,512 (1.9)	220 (0.1)	830 (0.4)	980 (1.9)	340 (1.2)
768 (5.3)	3,384 (2.6)	2,420 (0.8)	640 (0.3)	110 (0.2)	480 (1.6)
862 (6.0)	3,840 (2.9)	1,590 (0.5)	1,340 (0.7)	670 (1.3)	490 (1.7)
555 (3.9)	1,872 (1.4)	1,170 (0.4)	1,000 (0.5)	230 (0.4)	340 (1.2)
941 (6.5)	5,696 (4.3)	1,700 (0.6)	1,650 (0.9)	1,010 (1.9)	420 (1.4)
318 (2.2)	2,112 (1.6)	17,700 (6.1)	6,300 (3.3)	770 (1.5)	510 (1.7)
686 (4.8)	4,232 (3.2)	33,400 (11.5)	11,400 (6.0)	2,450 (4.6)	470 (1.6)
608 (4.2)	2,728 (2.1)	68,000 (23.5)	93,700 (49.7)	4,790 (9.1)	3,050 (10.4)
1,092 (7.6)	7,400 (5.6)	128,000 (44.2)	21,200 (11.2)	7,320 (13.9)	2,640 (9.0)
937 (6.5)	3,904 (2.9)	1,910 (0.7)	2,300 (1.2)	80 (0.2)	280 (1.0)
42 (0.3)	440 (0.3)	[c]	[c]	[c]	[c]
361 (2.5)	2,656 (2.0)	2,350 (0.8)	1,040 (0.6)	20 (0)	70 (0.2)
732 (5.1)	3,624 (2.7)	640 (0.2)	1,060 (0.6)	40 (0.1)	140 (0.5)
1,467 (10.2)	8,376 (6.3)	1470 (0.5)	1,900 (1.0)	80 (0.2)	260 (0.9)
173 (1.2)	2,936 (2.2)	610 (0.2)	920 (0.5)	50 (0.1)	40 (0.1)
948 (6.6)	3,408 (2.6)	560 (0.2)	540 (0.3)	10 (0)	80 (0.3)
265 (1.8)	304 (0.2)	790 (0.3)	0 (0)	0 (0)	0 (0)
662 (4.6)	4,944 (3.7)	1,360 (0.5)	490 (0.3)	330 (0.6)	1,180 (4.0)
510 (3.5)	912 (0.7)	830 (0.3)	320 (0.2)	100 (0.2)	6,720 (23.0)
481 (3.3)	4,728 (3.6)	13,700 (4.7)	8,820 (4.7)	620 (1.2)	280 (1.0)
14381	13,2456	289,720	188,450	52,760	29,190
14.4	132	290	188	53	29
1,307	7,208	[c]	[c]	[c]	[c]

[a]Value for the concentration of each chemical is in micrometer (μm), followed (in parentheses) by the concentration expressed as a percentage of the total free amino acids, to one decimal place.

[b]Values from Carr (1988)

[c]Not measured

the most abundant FAA in cephalopod inks. *O. bimaculoides* is unusual in that its taurine content is low and no single FAA dominates. Ammonium levels in cephalopod inks are also in the low millimolar range.

The major FAA in molluscan inks are those to which their major predators—crustaceans and fish—are sensitive, and their concentrations are well above response threshold, in many cases higher than response saturation. This is true of both the antennular (olfactory) and mouthpart (gustatory) chemoreceptor neurons of crustaceans (Voigt and Atema 1992; Derby 2000). Taurine and ammonium are noteworthy, as they are in such high concentrations in all of the molluscan secretions, and crustaceans have specialized receptor systems for them and are sensitive to submicromolar concentrations. Fish olfactory and gustatory systems are likewise highly sensitive to the FAA in these secretions, and have specialized receptors for several of them (Caprio 1988). These observations are consistent with the potential function of these secretions as sensory disruptors of molluscan predators. Furthermore, *A. californica* ink and opaline have much higher levels of TFAA (310–404 and 12–15 mM, respectively) compared to *A. californica* hemolymph and mucus (0.7 and

0.6 mM FAA, respectively). This finding is consistent with the idea that the FAA of sea hare ink and opaline are not reflective of the composition of other fluids or secretions of sea hares, but are more concentrated by up to several orders of magnitude, thus further supporting the idea that ink and opaline have evolved as a chemical defense.

Phagomimicry requires that the secretions contain chemicals in absolute and relative concentrations sufficient to mimic the food of predators. The molluscan secretions contain the same FAA as food, and they are generally present at similar absolute concentrations. TFAA in ink and opaline of *A. californica* and *A. dactylomela* (12–448 mM) and the inks of cephalopods (0.5–132 mM TFAA) are within or close to the range of TFAA in tissues of typical food of fish and crustacean predators (crab, shrimp, oyster, and mullet: 29–290 mM). This comparison of the FAA compositions of molluscan secretions and food tissues, together with behavioral results showing that an artificial mixture of *A. californica* ink or opaline with the FAA composition shown in Table 1 evokes feeding behaviors of the predatory spiny lobster *Panulirus interruptus* (Kicklighter et al. 2005), shows that molluscan secretions can be food-like in their TFAA.

Multidimensional scaling (MDS) was used to compare the relative FAA compositions of molluscan secretions with each other and with the prey species (Fig. 1). This analysis reveals several features of these secretions and tissues. First, different batches of the same secretion have similar proportions of FAA. *A. californica* ink samples Si1 and Si2, each representing samples pooled from 10–15 wild-caught animals, are similar to each other. A similar trend is seen for *A. californica* opaline samples So1 and So2. This shows relative constancy in the proportion of FAA for groups of animals collected at a different time and place. Second, secretions of a particular type (e.g., sea hare opaline, sea hare ink, or cephalopod ink) tend to be more closely grouped to each other, and thus have FAA composition more similar to each other than to those in a different type of secretion or tissue. The sea hare opalines (especially So2/So4) are clustered close to each other, the ink of three species of wild-caught sea hares (Si1, Si2, Si4) are clustered, and five of the six cephalopod inks (C1–C4, C6) are clustered. This supports the idea that although molluscan defensive secretions have quantitative and qualitative compositional similarities to food, the different molluscan secretions (molluscan opaline vs. molluscan ink, sea hare ink vs. cephalopod ink) have some differences in their FAA compositions. Third, whereas all of these molluscan secretions are similar to food in having high absolute concentrations of TFAA, the relative compositions (i.e., FAA proportions) of some molluscan secretions are more similar than others to food of their predators. All of the sea hare inks (Si1–Si4) and five of the cephalopod inks (*S. officinalis* C1, *L. pealei* C2, *L. brevis* C3, *D. gigas* C4, *O. vulgaris* C6) group close to each other and to oyster *Crassostrea virginica* (T3) and mullet *Mugil cephalus* (T4), indicating their FAA proportions are most similar. Sea hare inks and tissues from oyster and mullet also have some compositional similarity to the sea hare opalines, although less so than to each other. Relatively different in composition from all of these are sea hare hemolymph (Sf1), *Callinectes sapidus* muscle (T1), *Penaeus duorarum* muscle (T2), and *O. bimaculoides* ink (C5), and the equidose mixture (E). Thus, this MDS analysis, together with the absolute concentrations of FAA, shows that all molluscan secretions have FAA compositions highly similar to that of food, with some molluscan inks being more similar to food than are others.

Taurine, lysine, alanine, glycine, and glutamine contribute most to the compositional differences in the secretions and tissues in Fig. 1, as indicated by a linear multiple regression model of the MDS data ($P<0.001$) (Kruskal and Wish 1978). The concentration of taurine is relatively high in sea hare ink and opaline and in cephalopod inks, and relatively low in sea hare mucus and hemolymph and the crustacean muscle tissues. (The

exception is *O. bimaculoides*, which is not dominated by any component and thus is compositionally similar to the equidose mixture, E.) Regarding the other FAA in relatively high concentrations, lysine is high in sea hare opaline compared to all the other secretions and tissues. Alanine is relatively high in sea hare ink and cephalopod ink but not in sea hare opaline. Glutamine is absent in sea hare opaline but present in other secretions and food tissues. Glycine is relatively high in sea hare mucus and in the four food tissues.

The FAA composition of the molluscan defensive secretions and the sensory abilities of predators of these molluscs suggest that these secretions have the potential to function as chemical defenses through mechanisms that may include sensory disruption and/or phagomimicry. The focus of our paper is FAA and ammonium, but other chemicals in these secretions may also have defensive properties. A combination of mechanisms may enhance the effectiveness of the secretions (Kicklighter et al. 2005). For example, ammonium can be an inhibitor of feeding behavior of crustaceans (Zimmer-Faust 1987) and thus might also function as a deterrent in addition to its role as a sensory disruptor. Our demonstration of the presence in cephalopod ink of millimolar concentrations of feeding

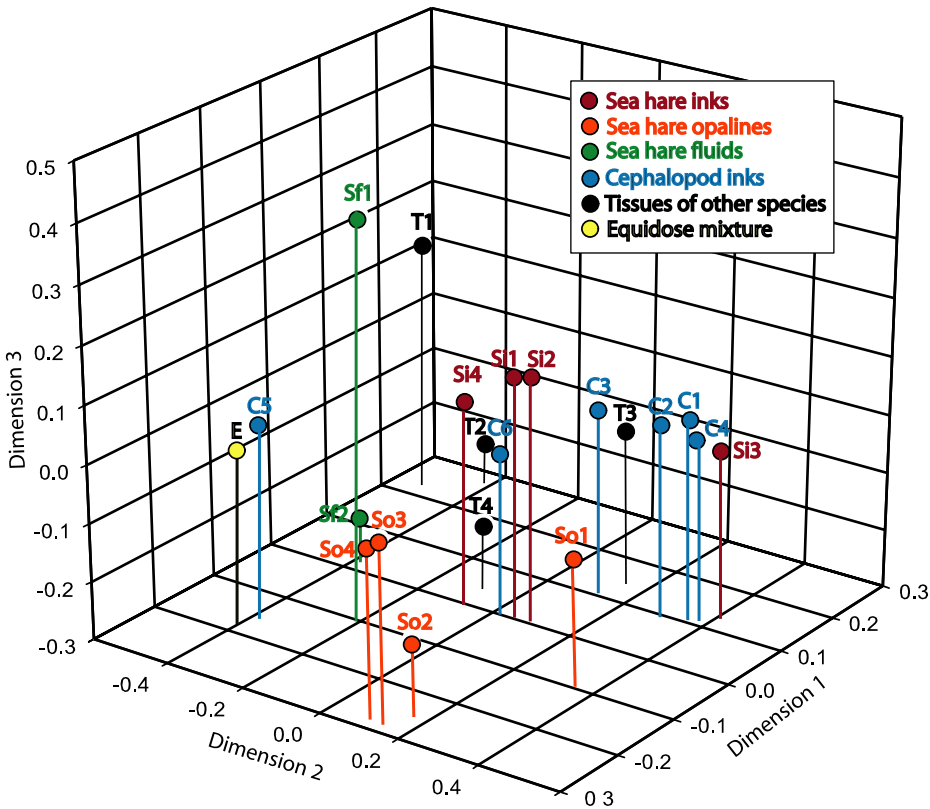


Fig. 1 Degree of similarity in the chemical composition of molluscan secretions and fluids, and food of predators of molluscs, based on multidimensional scaling. The identities of the secretions and tissues associated with the labels in this figure are coded according to type (e.g., Si=sea hare inks, So=sea hare opalines, C=cephalopod inks, T=food items), and the identity of each of the individual secretions and tissues (e.g., Si1, C4) are given in Table 1 and Methods and Materials. The values on the dimensions are derived from the analysis and are not strictly FAA concentration. See text for an explanation of the MDS analysis and results

chemostimulants of fish raises the possibility that cephalopod ink might have convergently evolved antipredatory effects through a combination of chemical mechanisms that may include sensory disruption or phagomimicry. Confirmation awaits behavioral testing.

Acknowledgments We thank Jelle Atema (Boston University), Tom Capo (NIH/University of Miami National Resource for *Aplysia* Facility), William Carr (University of Florida), William Gilly (Stanford University), Arie Sitthichai Mobley and Mary Lucero (University of Utah), Richard Taylor (University of Auckland), Leigh Walsh (National Resource Center for Cephalopods), and Michiya Kamio, Ko-Chun Ko, and Shkelzen Shabani (Georgia State University) for assistance with collection of secretions. Supported by grants from NSF (IBN/9876754, 0324435, 0322773, 0614685) and the GSU Brains & Behavior Program.

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J Chem Ecol (2007) 33:1115–1126
DOI 10.1007/s10886-007-9256-7

Nematicidal Resorcylides from the Aquatic Fungus *Caryospora callicarpa* YMF1.01026

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Received: 30 October 2006 / Revised: 19 January 2007 / Accepted: 23 January 2007 /
Published online: 29 March 2007
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Abstract This study investigated metabolites with activities against plant parasite nematodes from the fresh-water fungus *Caryospora callicarpa* YMF1.01026. We obtained three novel tetradecalactone metabolites, caryospomycins **A**–**C**, with such activities. The chemical structures of these were determined through NMR spectroscopic analysis and were found to belong to the 14-membered macrolides with a fused 1,2-dimethoxy-4-hydroxybenzene ring, a rare structure among the resorcylides. In the *in vitro* tests, all three compounds exhibited moderate killing activity against the nematode *Bursaphelenchus xylophilus*. To our knowledge, this is the first report of secondary metabolites in the aquatic fungal genus *Caryospora*.

Keywords *Caryospora carllicarpa* · Freshwater fungi · Nematicidal · Resorcylide

Introduction

The annual global loss in agriculture due to damages by plant-parasitic nematodes has been estimated as US $100 billion worldwide (Sasser and Freckman 1987). To reduce economic loss, several procedures for nematode control have been developed that use biological control agents and organic amendments. However, none of the procedures is ideal, and

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there is an urgent need for alternative, environmentally friendly measures, including natural nematocidal compounds (Noling and Becker 1994). One way to search for such compounds is to screen naturally occurring fungi. Fungi are important sources of naturally occurring antibiotics and pesticides. In addition, fungal secondary metabolites often have low plant and human toxicity and can be easily biodegraded (Siddiqui and Mahmood 1996). Compounds with nematicidal activity include alkaloids, peptides, terpenes, and fatty acids (Anke and Sterner 1997; Dong et al. 2005a, b, 2006). Omphalotin A, a cyclic dodecapeptide isolated from *Omphalotus olearius* (Mayer et al. 1997; Sterner et al. 1997), under *in vitro* conditions, out-performs known nematicides such as ivermectin in both potency and selectivity (Mayer et al. 1999).

These findings prompted us to conduct an evaluation of other little characterized members of freshwater fungi. Specifically, we were interested in the extracts of strain YMF1.01026 that belongs to species *Caryospora callicarpa* (Cai et al. 2002). This strain has shown high nematicidal activities towards the pine wood nematode, *Bursaphelenchus xylophilus* (Dong et al. 2004). In addition, there is so far no record of any previous investigation of the chemistry of this genus. The genus belongs to subfamily Zopfiaceae of the Dothideomycetidae and includes 15 species (*Caryospora australiensis*, *C. callicarpa*, *Caryospora cariosa*, *Caryospora coffeae*, *Caryospora langloisii*, *Caryospora lichenopsis*, *Caryospora mangrovei*, *Caryospora masonii*, *Caryospora minima*, *Caryospora minor*, *Caryospora nuclearia*, *Caryospora olearum*, *Caryospora phyllostachydis*, *Caryospora putaminum*, and *Caryospora striata*). In ths study, we report the bioassay-guided fractionation of extracts of *C. carllicarpa* YMF1.01026 that were tested against pine wood nematode, *B. xylophilus*.

Methods and Materials

Culture and Fermentation of C. callicarpa YMF1.01026 The fungal strain *C. callicarpa* YMF1.01026 was initially isolated from a submerged woody substrate collected from a freshwater habitat in Yunnan Province, China. This strain is deposited in the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Yunnan Province, the People's Republic of China (culture collection number YMF1.01026). The strain was maintained on PDA medium (potato 200 g, sucrose 20 g, agar 18 g, and water 1,000 ml) and was grown on wheat at 26°C for a period of 30 d before being processed for extracts.

Extraction and Isolation of Compounds Mycelial cultures were lyophilized and extracted with CH_3OH. The CH_3OH solution, after concentrated through vacuuming, was sequentially extracted ×3, each with petroleum ether and EtOAc. The combined EtOAc solution, upon evaporation, yielded a deep-brown syrup (~3.2 g). The syrup had *in vitro* nematicidal activity against pine wood nematodes *B. xylophilus*. This syrup was loaded onto a silica gel column [200 g Silica gel G (200–300 mesh), 3.6 cm i.d.×150 cm] and eluted by using solvent mixtures containing petroleum ether (bp 60–90°C)-EtOAc with increasing polarities (95:5 to 10:90). The resulting fractions were monitored by TLC (Silica gel G, 0.25-mm film thickness; Qingdao Marine Chemical Ltd., Qingdao, China) and reduced to one active fraction, **FV** (430 mg). The **FV** fraction, obtained on elution with petroleum (bp 60–90°C)-EtOAc (30:37), was further separated on a Sephadex LH-20 gel column and eluted with CH_3COCH_3 to yield pure **1** (16 mg), **2** (11 mg), and **3** (8 mg), respectively (Fig. 1).

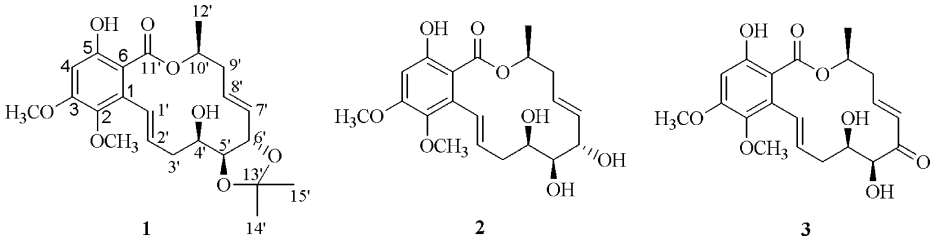


Fig. 1 Structures of caryospomycin **A**–**C** (**1**–**3**)

Identification of Compounds The structures of caryospomycins isolated from the cultures of *C. carllicarpa* YMF1.01026 were determined by spectroscopic analysis. Infrared (IR) spectra were obtained in KBr pellets with a Bio-Rad FTS-135 spectrophotometer (Bio-Rad, Richmond, CA, USA). UV spectra were taken on a Shimadzu double-beam 210A spectrophotometer (Shimadzu, Kyoto, Japan). Optical rotations were measured with a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). MS was performed on an Autospec-3000 spectrometer (VG, Manchester, England). Nuclear magnetic resonance (NMR) spectra were recorded on DRX-500 NMR (Bruker, Karlsruhe, Germany) spectrometers, with TMS as an internal standard and coupling constants were represented in Hertz.

Caryospomycin A (**1**): white amorphous powder (CH_3COCH_3); [α]+ 62.1° (CH_3OH; *c* 0.31); UV (CH_3OH) λ_{max} (log ε) 320.2 (4.01), 267.0 (4.22), 229.8 (4.70), 204.4 (4.71) nm; IR (film) ν_{max} 3,441, 2,959, 2,931, 2,875, 1,728, 1,648, 1,598, 1,472, 1,448, 1,381, 1,360, 1,316, 1,246, 1,224, 1,169, 1,126, 1,057, 1,041, 1,018, 969, 834, 743, 606; FABMS *m/z* (rel. int) 433$[M\text{-}H]^+$(3), 339 (40), 325 (100), 311 (60), 281 (42), 255 (5); EIMS *m/z* (rel. int) 434 $[M]^+$ (58), 416 $[M\text{-}18]^+$ (1), 372 (10), 296 (14), 267 (15), 249 (38), 223 (60), 221 (88), 219 (100), 205 (98), 195 (45), 193 (42), 167 (25), 109 (50), 95(58), 55(42); HRMS (ESI-TOF) *m/z*: 457.1844 $[M+Na]^+$ (calcd for $C_{23}H_{30}O_8Na$, 457.1838).

Caryospomycin B (**2**): white amorphous powder (CH_3COCH_3); [α]+ 30.0° (CH_3OH; *c* 0.76); UV (CH_3OH) λ_{max} (log ε) 319.0 (3.83), 267.2 (4.01), 225.4 (4.54) nm; IR (film) ν_{max} 3,424, 2,929, 2,856, 1,726, 1,648, 1,597, 1,474, 1,447, 1,383, 1,358, 1,308, 1,248, 1,226, 1,202, 1,169, 1,136, 1,063, 1,021, 965, 887, 610; FABMS *m/z* (rel. int) 393$[M\text{-}H]^+$(100); HRMS (ESI-TOF) *m/z*: 393.1554 $[M\text{-}H]^-$ (calcd for $C_{20}H_{25}O_8$, 393.1549).

Caryospomycin A (**3**): white amorphous powder (CH_3COCH_3); [α]+ 58.1° (CH_3OH; *c* 0.54); UV (CH_3OH) λ_{max} (log ε) 320.3 (3.97), 267.0 (4.07), 225.3 (4.60) nm; IR (film) ν_{max} 3,431, 2,939, 2,865, 1,728, 1,648, 1,598, 1,472, 1,448, 1,381, 1,360, 1,306, 1,245, 1,225, 1,200, 1,166, 1,059, 1,021, 969, 854, 609; FABMS *m/z* (rel. int) 391$[M\text{-}H]^+$(100); HRMS (ESI-TOF) *m/z*: 391.1403 $[M\text{-}H]^-$ (calcd for $C_{20}H_{23}O_8$, 391.1393).

Nematode Culture and Nematicidal Study Nematicidal activity was determined by using a microtiter plate assay as described previously (Dong et al. 2004). The test organism was the pine wood nematode *B. xylophilus*. This species has been maintained in our laboratory and used as a model target for *in vivo* nematocidal assays. For this assay, *B. xylophilus* was first grown on PDB agar media containing a strain of the fungus *Botrytis cinerea* in disposable Petri dishes prewetted with 2–4 ml of physiological saline. Cultures were then stored at room temperature and subcultured before assay. The assay was conducted in Corning polystyrene 96-well plates. Nematodes were added to 1 ml physiological saline in a scintillation vial and diluted until the nematode counts were 20–25 in a 48-μl aliquot. A solution (48 μl) containing nematodes was delivered to each of three wells for each

treatment. Two microliters of DMSO (5%) or DMSO (5%) plus the test compounds were added to each well. Plates were covered, parafilmed, and kept in a humid chamber. The numbers of live and dead nematodes were counted under a binocular microscope after various incubation times. Toxicity was inferred by using the mean percentage of dead organisms. Nematodes were considered dead if they gave no response to physical stimuli such as mechanical stirring or pricking with a pointed needle.

Statistical Analysis To evaluate nematicidal activity of compounds at different concentrations of 50, 80, 100, and 200 ppm, and different exposure times of 12, 24, and 36 hr, data were subjected to independent sample *F* test using ANALYZE (SPSS/version11.0 software, USA). Data on proof mortality were changed to $\sin^{1/2}$(M) before analysis.

To describe nematicidal effects of compound against *B. xylophilus*, LC_{50} was calculated according to probit analysis (Sporleder et al. 2005). Regression analyses were also conducted by SPSS for linear model. Data on proof mortality of nematodes were transformed into probit value, and concentrations (C) of compound were also changed to $\log_{10}$ (C) before analysis.

Results and Discussion

The molecular formula of caryospomycin A (**1**) was determined as $C_{23}H_{30}O_8$ by HRMS (ESI-TOF) and ^{13}C NMR data, indicating the presence of nine unsaturated bonds in the molecule. The IR (KBr) spectrum showed absorptions at 3,441, 1,728, 1,648, 1,598, 1,472, 1,448, 1,381, 1,360, 1,316, 1,246, 1,224, 1,169, 1,126, 1,057, 1,041, 1,018, 969, 834, 743, and 606 cm^{-1} that were attributable to hydroxyl, ester carbonyl, olefin, ether functions, and the aromatic ring. The UV spectrum maxima near 305, 265, and 220 were typical of the 4-methoxy resorcylic acid lactone macrolide chromophore present in the radicicol derivatives (Mirrington et al. 1964; Nair et al. 1981). The ^1H NMR spectrum (Table 1) showed signals attributable to the presence of one secondary methyl [δ_H 1.44 (3H, d, J=6.4 Hz)], two tertiary methyls [δ_H 1.34 (3H, s, acetonide), 1.28 (3H, s, acetonide)], and two oxymethyls [δ_H 3.88 (3H, s), and 3.56 (3H, s)]. An exchangeable 1H singlet at δ_H 11.1 was assigned to the chelated phenolic hydrogen (Nair et al. 1981; Isaka et al. 2002). Additional resonances were observed in the ^1H and ^{13}C NMR spectra for two *trans* 1,1-disubstituted double bonds [δ_H 6.75 (1H, d, J=15.9 Hz), 5.96 (1H, dt, J=15.6, 5.7, 3.5 Hz), 5.55 (1H, dd, J=8.6, 15.4 Hz), and 5.99 (1H, dt, J=15.4, 5.3, 3.5 Hz); δ_C 127.2 (d), 131.5 (d), 133.9 (d), and 129.4 (d)], and four oxymethine carbons [δ_H 3.86 (1H, dd, J=8.0, 2.0 Hz), 4.15 (1H, m), 4.58 (1H, t, J=8.3 Hz), 5.41 (1H, m); δ_C 69.2 (d), 72.0 (d), 76.1 (d), 82.3 (d)]. The ^{13}C NMR spectra also revealed resonances consistent with one lactone carbonyl [δ_C 171.7 (s)], one acetonic carbon [δ_C 108.7 (s)], and one penta-substituted benzene ring [δ_H 6.46 (1H, s); δ_C 100.2 (d), 105.1 (s), 133.6 (s), 141.4 (s), 159.5 (s), 160.8 (s)]. From this analysis, coupled with the degrees of unsaturations (9), it became clear that **1** was a resorcylic macrolide with two *trans*-disubstituted olefins.

The HMQC analysis revealed the assignment of each direct C–H bonding in **1** as summarized in Table 1. The ^1H–^1H correlations obtained from the ^1H–^1H COSY exhibited one big spin system, substructure **1a**, as depicted in Figs. 2 and 3. The HMBC correlations from O**H** at C-5 (δ_H 11.1, s) to C-6 (δ_C 105.1), C-5 (δ_C 160.8), C-4 (δ_C 100.2), from the aromatic proton H-4 (δ_H 6.46, s) to C-6 (δ_C 105.1), C-5 (δ_C 160.8), C-3 (δ_C 159.5), C-2 (δ_C 141.4), and C-11′ (δ_C 171.1), from one oxymethyl OC**H**$_3$-3 (δ_H 3.88, s) to C-4 (δ_C 100.2), C-3 (δ_C 159.5), and from the second oxymethyl OC**H**$_3$-2 (δ_H 3.56, s) to C-2

Table 1 ^{13}C and ^{1}H NMR data of compounds **1**–**3**[a] (Acetone-d_6)

Number	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	133.6 s		134.3 s		133.5 s	
2	141.4 s		141.0 s		141.1 s	
3	159.5 s		159.2 s		159.4 s	
4	100.2 d	6.46 (s)	100.1 d	6.45 (s)	100.4 d	6.48 (s)
5	160.8 s		160.0 s		160.3 s	
6	105.1 s		105.8 s		104.2 s	
1′	127.2 d	6.75 (d, 15.9)	126.0 d	6.67 (d, 16.0)	126.0 d	6.61 (d, 15.9)
2′	131.5 d	5.96 (dt, 15.6, 5.7, 3.5)	133.3 d	5.95 (dt, 15.6, 6.2, 3.0)	133.5 d	5.91 (dt, 15.6, 6.3, 3.0)
3′	38.0 t	2.71 (H_α, m)	37.9 t	2.41 (H_β, m), 2.56 (H_α, m)	39.0 t	2.36 (H_β, m), 2.91 (H_α, m)
4′	69.2 d	4.15 (m)	73.5 d	3.73 (m)	75.0 d	3.87 (m)
5′	82.3 d	3.86 (dd, 8.0, 2.0)	78.6 d	3.53 (dd, 8.2, 2.0)	79.8 d	4.52 (d, 3.5)
6′	76.1 d	4.58 (t, 8.3)	73.9 d	4.13 (t, 7.8)	198.0 s	
7′	133.9 d	5.55 (dd, 8.6, 15.4)	134.2 d	5.65 (dd, 7.6, 15.5)	132.3 d	6.43 (d, 15.4)
8′	129.4 d	5.99 (dt,15.4, 5.3, 3.5)	128.4 d	5.94 (dt, 14.2, 5.5, 3.9)	142.6 d	7.00 (dt, 15.5, 6.0, 3.5)
9′	37.8 t	2.50 (H_β, m), 2.56 (H_α, m)	37.4 t	2.54 (m)	38.2 t	2.80 (m)
10′	72.0 d	5.41 (m)	73.2 d	5.28 (m)	73.4 d	5.32 (m)
11′	171.7 s		171.9 s		171.8 s	
12′	19.3 q	1.44 (d, 6.4)	19.8 q	1.42 (d, 6.3)	19.8 q	1.48 (d, 6.2)
13′	108.7 s					
14′	27.1 q	1.34 (s)				
15′	27.4 q	1.28 (s)				
2-OCH3	60.1 q	3.56 (s)	60.1 q	3.54 (s)	60.1 q	3.57 (s)
3-OCH3	56.2 q	3.88 (s)	56.2 q	3.87 (s)	56.0 q	3.87 (s)
5-OH		11.1 (s)		11.2 (s)		11.3 (s)

[a] Assignments of the ^{13}C and ^{1}H signals were made on the basis of HMQC.

(δ_C 141.4) established one penta-substituted benzene ring, substructure **1b**, as depicted in Fig. 3. Furthermore, HMBC cross peaks of the olefinic proton at H-1′ (δ_H 6.75, d) with C-6 (δ_C 105.1), C-1 (δ_C 133.6), C-2 (δ_C 141.4), C-2′ (δ_C 131.5), and C-3′ (δ_C 38.0), the olefinic proton H-2′ (δ_H 5.96, dt) with C-3′ (δ_C 38.0), C-4′ (δ_C 69.2), C-1′ (δ_C 127.2), and C-1 (δ_C 133.6), the oxymethine proton H-10′ (δ5.41, m) with C-11′ (δ_C 171.7), C-12′ (δ_C 19.3), C-8′ (δ_C 129.4), and C-7′ (δ_C 133.9) established the reasonable connection patterns of C-1 with C-1′ and C-10′ with C-11′ through an oxygen atom, and permitted fragments **1a** and **1b** to be joined together. Finally, the HMBC correlations of two geminal methyl groups, C**H**$_3$-14′ and C**H**$_3$-15′ to C-13′ identified the occurrence of the acetonide moiety **1c**. Based on the ^{13}C NMR chemical shifts values (δ_C 27.1 and δ_C 27.4) of two geminal methyl

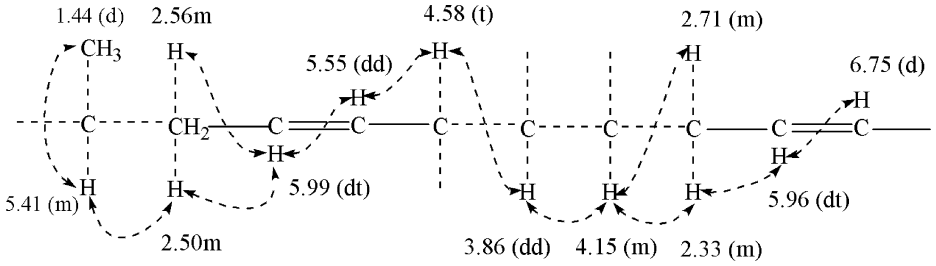


Fig. 2 Substructure **1a**, ^{1}H NMR data, and ^{1}H–^{1}H correlations (dashed arrows) of caryospomycin **A** (**1**)

groups, and the strong cross peak at H-5′ (δ_H 3.86, dd) with C**H**$_3$-15′ (δ_H 1.28, s) and the weak cross peak at H-6′ (δ_H 4.58, t) with C**H**$_3$-14′ (δ_H 1.34, s) in the NOESY spectrum, the acetonide carbon (C-13′, δ_C 108.7, s) was connected to C-5′ (δ_C 82.3) and C-6′ (δ_C 76.1) with *trans* stereochemistry (Adamczeski et al. 1988; Rychnovsky and Skalitzky 1990). Thus, the structure of caryospomycin **A** was established as **1**.

Both coupling constants between H-1′ (δ_H 6.75) and H-2′ (δ_H 5.96), H-7′ (δ_H 5.55) and H-8′ (δ_H 5.99) olefinic protons were measured as near 15 Hz, indicating the *E* geometry of the carbon–carbon double bonds in the diene at C-1′(2′) and C-7′(8′), because the *cis* coupling constant in comparable hypothemycin is 11 Hz (Nair and Carey 1980; Nair et al. 1981). The relative stereochemistries of C-10′ (δ_C 72.0), C-6′ (δ_C 76.1), C-5′ (δ_C 82.3), and C-4′ (δ_C 69.2) in **1** was deduced from the analysis of NOESY correlations with supporting information from vicinal coupling constants (Table 1). A NOESY experiment on **1** showed cross-peaks between H-1′ (δ_H 6.75)/$H_\beta-3'$(δ_H 2.33), $H_\beta-3'$(δ_H 2.33)/H-6′ (δ_H 4.58), H-6′ (δ_H 4.58)/C**H**$_3$-14′ (δ_H 1.34), H-4′(δ_H 4.12)/$H_\alpha-3'$(δ_H 2.33), H-4′(δ_H 4.12)/H-5′(δ_H 3.86), H-5′(δ_H 3.86)/C**H**$_3$-15′ (δ_H 1.28), H-7′ (δ_H 5.55)/$H_\beta-9'$(δ_H 2.50), H-7′ (δ_H 5.55)/$H_\alpha-9'$(δ_H 2.56), H-8′(δ_H 5.99)/$H_\alpha-9'$(δ_H 2.56), and $H_\alpha-9'$(δ_H 2.56)/H-10′(δ_H 5.41) (Fig. 4). This result demonstrated that $H_\alpha-3'$, H-4′, H-5′, $H_\alpha-9'$, C**H**$_3$-15′, and H-10′ were α-oriented, while $H_\beta-3'$, H-6′, C**H**$_3$-14′, $H_\beta-9'$, and C**H**$_3$-12′ possessed β-orientation. The 2.0 H-4′/H-5′ coupling constant and the almost 8 Hz H-5′/H-6′ coupling constant further supported the axial configurations for H-4′ and H-5′, as well as the equatorial configuration for H-6′. From the ^{1}H NMR, COSY, and NOESY spectra, a

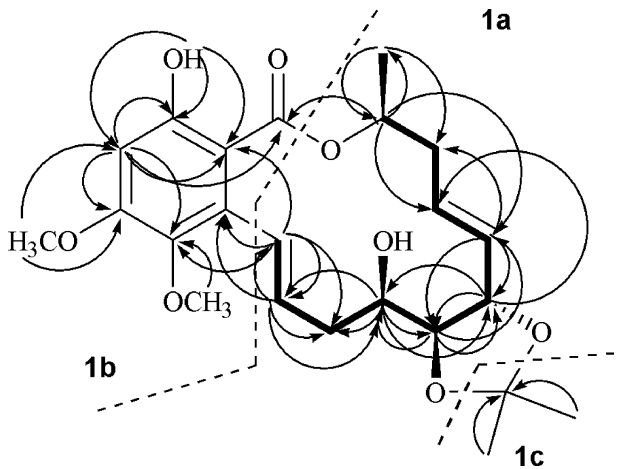


Fig. 3 Gross structure, ^{1}H–^{1}H correlations (bold lines), key HMBC correlations (plain arrows) of caryospomycin **A** (**1**)

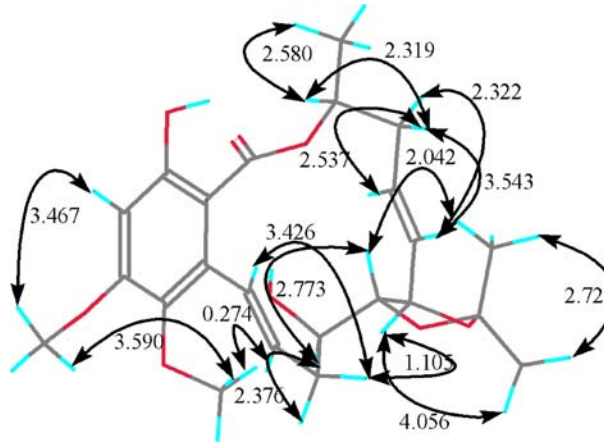


Fig. 4 Key NOESY correlations of **1** and corresponding interatomic distance (A), and some protons hidden for concision of the picture

computer-generated 3D structure of **1** was obtained by using the molecular modeling program, CS CHEM 3D V 4.0, with MM2 force-field calculations for energy minimization (Fig. 4). The calculated distances between $H-1'/H_\beta-3'$, $H_\beta-3'/H-6'$, $H-4'/H_\alpha-3'$, H-4′/H-5′, H-5′/C**H**$_3$-15′, $H-7'/H_\beta-9'$, $H-7'/H_\alpha-9'$, $H-8'/H_\alpha-9'$, and $H_\alpha-9'/H-10'$ were all less than 4.00 Å, except that the calculated distance between H-6′ and C**H**$_3$-14′ was slightly higher, at 4.056 Å. This high value is consistent with the observed weak cross peak at H-6′ with C**H**$_3$-14′ and the observed strong cross peaks from each of these proton pairs. Thus, the relative overall stereochemistry for compound **1** was deduced as 1′*E*, 4′*R**, 5′*S**, 6′*S**, 7′*E*, 10′*R**.

The molecular formula of caryospomycin B (**2**), $C_{20}H_{26}O_8$, was established from a high-resolution MS (ESI-TOF) measurement of the $[M-H]^-$ peak at *m/z* 393.1554, indicating the presence of eight unsaturated bonds in the molecule. The UV, IR, and NMR spectra were similar to those obtained for **1**, indicating that they were structurally related. The most striking differences in the NMR data between **1** and **2** compared were the disappearance of the acetonide moiety signals (δ_C 108.7, 27.1 q, and 27.4 q; δ_H 1.34 s and 1.28 s) in **2** but their presence in **1**. In the ^{13}C NMR data, signals of the C-5′ and C-6′ oxymethine carbons were noticeably shifted upfield from δ_C 82.3 and 76.1 in **1** to δ_C 78.6 and 73.9 in **2**, while the C-4′ oxymethine carbon signal was noticeably shifted downfield from δ_C 69.2 in **1** to δ_C 73.5 in **2** (Table 1). Corresponding differences were observed in the ^1H NMR data, in which signals of the oxymethine protons were shifted upfield from δ_H 4.15 (1H, m, H-4′), 3.86 (1H, dd, *J*= 8.0, 2.0 Hz, H-5′), and 4.58 (1H, t, *J*=8.3 Hz, H-6′) in **1** to δ_H 3.73 (1H, m, H-4′), 3.53 (1H, dd, *J*=8.2, 2.0 Hz, H-5′), and 4.13 (1H, t, *J*=7.8 Hz, H-6′) in **2**. These spectral changes were consistent with the absence of the acetonide moiety from C-13′ to C-15′ in **2**. Despite the spectral differences, however, the combined 2D NMR experiments showed that **2** had the same proton-proton and proton-carbon correlations as **1** throughout the entire molecule. Thus, the structure of **2** was determined to be a deacetonided derivative of **1**.

The molecular ion $[M]^+$ of caryospomycin C (**3**) is 2 mass units lower than compound **2**, and its molecular formula was determined as $C_{20}H_{24}O_8$ by HRMS (ESI-TOF) and NMR data. One noticeable difference in the ^{13}C NMR data between **2** and **3** was the addition of a ketone functionality at δ_C 198.0 in **3**, and the absence of an oxymethine carbon at this position, an observation suggesting that the oxymethine carbon of **2** was replaced by the ketone functionality in **3**. In addition, the ^{13}C chemical shifts of the C-7′/C-8′ double band and the C-4′, C-5′ oxymethines were also changed from δ_C 134.2 (CH, C-7′), 128.4 (CH,

C-8′), 73.5 (CH, C-4′), and 78.6 (CH, C-4′) in **2** to δ_C 132.3 (CH, C-7′), 142.6 (CH, C-8′), 75.0 (CH, C-4′), and 79.8 (CH, C-4′), respectively, in **3**. Corresponding differences were observed in the ^{1}H NMR data, in which signals of the olefinic and oxymethine protons were shifted downfield from δ_H 5.94 (dt, J=14.2, 5.5, 3.9 Hz, H-8′), 5.65 (dd, J=7.6, 15.5 Hz, H-7′), 3.53 (dd, J=8.2, 2.0 Hz, H-5′), 3.73 (m, H-4′) in **2** to 7.00 (dt, J=15.5, 6.0, 3.5 Hz, H-8′), 6.43 (d, J=15.4 Hz, H-7′), 4.52 (d, J=3.5 Hz, H-5′), 3.87 (m, H-4′) in **3**. Based on these spectral changes, the newly appearing ketone carbonyl was placed in between C-5′ and C-7′ neighboring the C-7′/C-8′ double band. This was confirmed by HMBC correlations between the protons at δ_H 6.43 (H-7′), 7.00 (H-8′), and 3.87 (H-4′), and the ketone carbonyl signal at δ_C 198.0. Thus, the structure of caryospomycin **C** was determined to be an analogue of **2** but with a different oxidation state at C-6′, as shown in Fig. 1.

Nematicidal Activity The nematicidal activities of caryospomycin **A**–**C** are shown in Table 2. Caryospomycin **A**–**C** displayed moderate nematicidal activities against *B. xylophilus*. Nematicidal effects varied with concentration and exposure time. Activity differed significantly between different exposure times of 12, 24, and 36 h at the same concentration (Table 3), and different concentration of 50, 80, 100, and 200 ppm at the same exposure time (Table 4). Probit value of proof mortality showed linear type of increase with increasing Log_{10} (concentrations). The LC_{50} values of compounds **1**-**3** against *B. xylophilus* were 103.1, 105.8, and 105.1 ppm, at 36 hr, respectively.

The study demonstrated the presence in *C. carllicarpa* YMF1.01026 of three new nematicidal metabolites, all of which belonged to the resorcylic 14-membered macrolide family. This family of compounds includes many well-characterized chemicals such as zearalenone isolated from *Gibberella zeae* and *Fusarium culmorum* (Stob et al. 1962; Richardson et al. 1985); radicicol (also named monorden) from *Chaetomium chiversii* (Turbyville et al. 2006), *Cylindrocarpon radicicola* (Evans and White 1966), *Monocillium nordinii* (Ayer et al. 1980), *Penicillium luteo-aurantium* (Nozawa and Nakafima 1979), *Neocosmospora tenuicristata* (Cutler et al. 1987), *Verticillim chlamydosporium* (Khambay et al. 2000), and *Humicola* sp. F02942 (Wicklow et al. 1998; Arai et al. 2003); monorcillins from *Monocillium nordinii* (Ayer and Pena-Rodriguez 1987; Ayer et al. 1980), *Diheterosporia chlamydosporia* (Espenshade and Calton 1979), *Humicola fuscoatra* NRRL22980 (Wicklow et al. 1998), *Humicola* sp. (Arai et al. 2003; Yamamoto

Table 2 Effect of caryospomycin **A**–**C** on the proof mortality of *B. xylophilus in vitro*

Compounds	Percent mortality (hr)	Concentrations (ppm)				LC_{50} (ppm)
		200	100	80	50	
1	12	14.5	7.9	6.8	4.4	1011.6
	24	57.1	40.3	29.7	20.1	164.7
	36	72.4	50.3	41.3	33.5	103.1
2	12	16.7	13.4	7.6	5.1	1255.6
	24	47.8	42.1	31.3	12.6	185.9
	36	67.5	54.7	50.1	23.7	105.8
3	12	18.8	16.1	8.3	4.4	620.3
	24	38.5	30.6	19.6	20.0	442.1
	36	64.2	57.8	50.7	25.5	105.1

Table 3 Influence of different exposure time (12, 24, and 36 hr) on the mortality of nematodes at each concentration of compounds **1**–**3**

Compounds	Concentrations (ppm)			
	200[a]	100	80	50
1	212.6*[b]	320.4*	166.797*	156.3*
2	602.4*	235.4*	273.4*	104.3*
3	489.5*	375.6*	468.1*	103.5*

*$P<0.001$

[a] Concentrations (mg l^{-1}) of the compound

[b] Values were *f* values from independent sample ANOVA

et al. 2003), and *Paraphaeosphaeria quadriseptata* (Wijeratne et al. 2004); pochonins from *Pochonia chlamidospora* (Hellwig et al. 2003); hypothemycin from *Hypomyces trichothecoides* (Nair and Carey 1980; Nair et al. 1981; Agatsuma et al. 1993); and aigialomycins from *Aigialus parvus* (Isaka et al. 2002). All of these compounds have been reported to have a wide range of biological activities such as antifungal, antitumor, antiprotozoan, antimalarial, antiviral, and antiparasitic functions. Among them, zearalenone and radicicol are probably the two most noteworthy. Specifically, zearalenone possesses anabolic, estrogenic, and antibacterial activities. Compounds exhibiting anabolic activities can be employed as cattle-growth stimulants (Lone 1997) as well as in treatments of menopausal and postmenopausal syndromes (Utian 1973). The compound radicicol exhibits various biological activities including antifungal, antibiotic, and antimalarial functions (with *in vivo* efficacy). In addition, radicicol inhibits various kinases and the Heat-shock protein Hsp 90 (Janin 2005). There have been over 200 publications and patents associated with this compound (Jayasuriya et al. 2005). Among its various activities, the inhibition against Hsp90 has attracted significant attention in recent years. Specifically, this compound is capable of suppressing the transformed phenotype caused by various oncogenes such as *src*, *ras*, and *raf*. These suppressive functions are linked to radicicol's high-affinity binding (20 nM) and inhibition of the Hsp90 molecular chaperone. This "antichaperone" activity might help reduce the level of oncogenic proteins, and could thus be of clinical interest. Consequently, the resorcylic 14-membered macrolides have been the subject of many preparative and biosynthetic studies (Nicolaou et al. 1998; Furstner et al. 2000; Burckhardt and Ley 2002; Yang and Danishefsky 2003; Moulin et al. 2005). Our finding that the widely distributed fresh water fungus *C. carllicarp* YMF1.01026 can

Table 4 Influence of different concentration (50, 80, 100, and 200 ppm) on the mortality of nematodes at each exposure time

Compounds	Exposure time (hr)		
	12[a]	24	36
1	19.1*[b]	78.3*	111.1*
2	33.6*	193.2*	166.0*
3	86.5*	72.8*	171.8*

*$P<0.001$

[a] Times of the exposure

[b] Values were *f* values from independent sample ANOVA

also produce resorcylic 14-membered macrolides may be of significant biomedical importance. Further studies are required to examine the resorcylide biosynthetic capacities of this fungus and to obtain sufficient quantities of caryospomycins **A**–**C** as well as their analogues to fully evaluate their biological activities.

The present study demonstrated that fungi inhabiting freshwater environments can produce nematicidal metabolites. The occurrence of nematicidal substance in freshwater fungi might be linked to their survival strategies. For example, nematicidal compounds could help fungi obtain nutrients (e.g., organic nitrogen compounds) from dead nematodes, similar to the proposed functions of these groups of compounds in some wood-inhabiting Basidiomycetes like *Pleurotus* species (Thorn and Barron 1984; Barron 1992). In freshwater ecosystems, submerged woody substrata constitute the main energy input source (Wong et al. 1998). Wood is, however, a substrate notably deficient in nitrogen, and thus the nitrogen utilized by freshwater fungi might need to come from other sources. Nematodes are cosmopolitan organisms and are adapted to live in both terrestrial and aquatic environments. They have been shown to be an integral part of various ecosystems, providing food and energy for small invertebrates or fungi (Dropkin 1980). With their high nitrogen contents, nematodes are considered to play an important role in providing nitrogen to other organisms in freshwater ecosystems. Several nematophagous fungi have previously shown to be associated with nematodes found in wood that was submerged in freshwater. These fungal species included *Dactylella ellipsospora* Grove (Hyde and Goh 1998) and *Dactylella aquatica* (Ingold) Ranzoni (Kane et al. 2002). Similarly, freshwater fungi producing nematicidal compounds might also obtain their nitrogenous nutrients from nematodes living in submerged wood. The ability for these fungi to produce nematicides that can kill nematodes and release nutrients could be highly advantageous for these fungi in their natural environments.

Acknowledgements This study was financially supported by the National Natural Science Foundation of China (NSFC30570059 and NSFC20562015), Yunnan Provincial Natural Science Foundation (2005C0005Q, 2005NG03 and 2005NG05), and the State Key Laboratory of Phytochemistry and Plant Resources in Western China, Kunming Institute of Botany, China. We are grateful to Dr. L. Cai for providing strain *Caryospora carllicarpa* YMF1.01026.

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J Chem Ecol (2007) 33:1127
DOI 10.1007/s10886-007-9296-z

LETTER FROM THE EDITOR-IN-CHIEF

John T. Romeo (For the Associate Editors and Editorial Board)

All of us are saddened by the passing of R. Milton Silverstein, one of the founding editors of the *Journal of Chemical Ecology*. Coming just a little over 2 years after the death of his friend and co-founder John Simeone, this is truly the end of an era. The following tribute by those who knew him well reminds us of the legacy he left, as well as his warmth, wit, and humility.

We commemorate his vision and accomplishments as a scientist, which will long inspire us. The Silverstein/Simeone award, which is awarded annually by the International Society of Chemical Ecology and funded by Springer Publishing, will continue to recognize scientists who are advancing the discipline of Chemical Ecology with innovative research.

Milt was also, and above all, a humanist, and we are forever grateful for the privilege of knowing him. It is with gratitude, respect, and affection that we dedicate this issue of the Journal to Milt.

J Chem Ecol (2007) 33:1128–1130
DOI 10.1007/s10886-007-9297-y

IN MEMORIAM

Robert Milton Silverstein 1916–2007

Dietland Muller-Schwarze · James H. Tumlinson · Francis X. Webster · David L. Wood

Published online: 16 May 2007
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On February 26, 2007, Robert Milton Silverstein died peacefully, surrounded by his family, at the age of 90. He was an internationally recognized expert on the chemistry of insect communication. His pioneering studies led to the development of the field of chemical ecology. Born in Baltimore, MD, he grew up in Tottenville, Staten Island. He earned a B.S. in Chemistry at the University of Pennsylvania and an M.S. and a Ph.D. at New York University. During WWII, Milt served in the Army Medical Corps and was sent to the Pacific Theater, where he rose to the rank of Captain. He married Olive Jorgenson in 1943. They began a family and, in 1948, moved to California. Milt worked for 21 years at the Stanford Research Institute (SRI), where he made significant contributions in analytical methods, chemical synthesis, and flavor and fragrance chemistry. While at SRI, he began his ground-breaking studies in chemical ecology and wrote the first edition of his textbook,

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Spectrometric Identification of Organic Compounds. This enduring textbook is now in its seventh edition and has been translated into five languages.

In 1962, Milt phoned the Department of Entomology and Parasitology at the University of California, Berkeley (UCB), to inquire about entomologists working on insect behavior. What motivated him to make that phone call to UCB and other universities? He was ahead of the times. He recognized the significance of Butenandt's landmark identification of the silkworm moth sex attractant in 1959. At that time, synthetic organic pesticides were being used indiscriminately against insects, mites, and nematodes in the US and throughout the world. As a consequence, pest resistance, resurgence, and replacement with new pests became a central theme in pest control. In those early days, Milt had concluded that, through the application of their newly developing techniques in microchemistry, naturally occurring compounds that control insect behavior and development could be identified and thus be made available to reduce pest-caused damage in an environmentally friendly way. He vigorously pursued this objective throughout his career, as can be seen from the long list of invertebrate pests that he investigated. His great versatility and wide-ranging interests are further highlighted by his collaborative investigations of vertebrate behavior.

He collaborated with David Wood to isolate and identify the first beetle pheromone. This was also the first multicomponent pheromone to be identified, and they were the first to demonstrate the importance of using a complete blend of pheromone components. Simultaneously, they had discovered synergism among the three active terpene alcohols isolated from the attractive substrate (frass or fecal material). None of the compounds were active alone! They rushed to the field to test two of these compounds that together elicited an attractive response in the lab. Milt could be seen picking the little (3–4-mm) beetles out of the talcum powder-like dust around the trap and tossing them onto the trap. He was convinced that they were "near misses" and were, in fact, responding to that treatment. With or without the near misses, the differences were obvious. But, they had trapped a different species from the one producing the compounds that were being tested! When all three compounds were released the next day, they trapped the species that produced these compounds, but not the other species. They were amazed by these unexpected results! Milt published one of his favorite quotes a few years later: "To him who in the love of Nature holds Communion with her visible forms, she speaks A various language..." *Thanatopsis*.

During experiments in 1965 near Yosemite National Park, Milt learned the rigors of field research. When the team arrived in the field site they soon realized that they had forgotten the release devices. Milt offered to stay behind and watch over the traps. He lay down in the tall grass to take a nap, as he was also recovering from a small dose of the "killer grape" that had been enjoyed the previous evening. Suddenly, he was awakened by an order to stand up slowly and identify himself. As he opened his eyes, he stared up at the barrel of a sawed-off shotgun aimed at his head by a deputy sheriff from Madera County. He said that what went through his head was, "I am a chemist employed by the SRI in Menlo Park, California, and that I am in this brush field testing bark beetle pheromones...No!...He will never believe this story!" Milt's employee card saved the day.

Milt was invited to lecture in France, but his hosts asked if he would be willing to deliver it in French! Always rising to the challenge, Milt sat in his office for weeks memorizing his speech. When asked questions after his talk, his French colleagues quickly realized what an accomplishment he had made to accommodate their request. He disdained paperwork. On one occasion when he was out of town, a secretary tried to find a portion of a research proposal in his files. She returned empty-handed, exclaiming: "He keeps nothing in his file cabinets. They are empty!" Besides being an outstanding scientist, Milt also

relaxed in nature. He and Olive shared many memorable hours in the woods with friends, watching beavers, skiing, hiking, and gathering wild apples.

Milt wrote the definitive papers on the role of chirality in insect pheromones. He and his colleagues demonstrated the importance of host tree compounds in synergizing bark beetle pheromones and identified and synthesized the pheromones of over 20 species of beetles, several ticks, moths, and ants, as well as mammals like the deer and pronghorn antelope. His pioneering work on pheromones provided the knowledge and the tools for successful monitoring and control programs for insect pests of forests, stored products, and agricultural crops. As an example, the pink bollworm moth sex pheromone, which he played a critical role in identifying, is used successfully for control of this important pest of cotton, thus avoiding the use of pesticides on thousands of acres.

In 1969, Milt and his family moved to Syracuse, NY, where he was appointed Professor of Chemical Ecology at the State University of New York College of Environmental Science and Forestry (ESF), the first position of its kind in the world. At ESF, he focused on developing the field of chemical ecology, publishing more than 200 research papers and seven books over his career. He was a founding member of the International Society of Chemical Ecology and helped to establish its Journal. He served, along with John Simeone, as editor until 1994. Milt is honored by the society with its annual presentation of the Silverstein–Simeone Award, which is given to a scientist for outstanding lifetime contributions. He also received many other scientific honors, including the Bussart Memorial Award from the Entomological Society of America and the first Silver Medal of the International Society for Chemical Ecology, culminating in his election to the National Academy of Sciences. He was predeceased by his son, Roy; his daughter, Amy; and his brother, Fred. He is survived by his wife, Olive; son Paul and his wife, Elizabeth; grandchildren Brian, Ilana, and Nava; great-grandson Matthias; several nieces and nephews; and other close family and friends. A memorial service was held March 17, 2 to 4 P.M. at the First Unitarian Universalist Society of Syracuse, 250 Waring Road, Syracuse. Memorial contributions may be made to the Alzheimer's Association, 441 W. Kirkpatrick St., Syracuse, NY, 13204, and Greenleaf Funeral Home, Inc.

We are fortunate to have traveled on life's path together. Thank you Milt, and, in his understated words of praise, "good show."

J Chem Ecol (2007) 33:1131–1148
DOI 10.1007/s10886-007-9284-3

Determining Larval Host Plant Use by a Polyphagous Lepidopteran Through Analysis of Adult Moths for Plant Secondary Metabolites

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Received: 7 November 2006 / Revised: 23 February 2007 / Accepted: 14 March 2007 /
Published online: 10 May 2007
© Springer Science + Business Media, LLC 2007

Abstract Many polyphagous insect species are important economic pests on one or more of their crop hosts. For most important insect pests, the common crop hosts are well-known, but knowledge of weedy and unmanaged hosts is limited. Furthermore, the relative contribution of different hosts to local and regional populations has rarely been ascertained because this requires having some way to determine which plant hosts are the source of the adult moths observed ovipositing in a crop field at a given place and time. One way of determining the larval host of polyphagous pest species is to analyze for several plant-derived chemicals that are each specific to a different small set of related plant species and are preserved in detectable amounts in adult moths. In this paper, we describe novel methods for analyzing adults of the polyphagous lepidopteran, the tobacco budworm (TBW) *Heliothis virescens* (F.), for plant secondary metabolites, specifically cotinine and gossypol, which are diagnostic for larval feeding on tobacco and cotton, respectively. Cotinine was extracted from individual TBW moths with acetic acid and methanol, then concentrated and analyzed directly by gas chromatography/mass spectrometry (GC/MS). The same moths then were analyzed for bound gossypol by creating a Schiff's base that used aniline, and the resulting dianilino–gossypol complex was quantified using high pressure chromatography coupled with a triple quadrupole mass spectrometer (MS) as the detector. Based on analysis of standards, the detection limit for the cotinine was less than 1.5 ppb by dry weight. Comparable standards were not available for the gossypol derivative so a quantitative limit of detection could not be calculated. When TBW moths reared on known hosts were analyzed for gossypol and/or cotinine, all of the moths reared on tobacco or cotton were correctly identified, although some false positives were recorded with the gossypol method. Analysis of TBW moths of various ages and at various lengths of time after death determined that a significant gossypol signal was detectable in all moths reared on cotton. TBW moths collected from the vicinity of cotton fields in July and August in North Carolina also were analyzed. A much larger portion of the moths were derived from tobacco (6.7–46.4%) than from cotton (0–3.6%) in both months. Thus, these methods can be reliably used to estimate the proportion of TBW derived from noncotton host plants in

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populations trapped around Bt cotton fields, thereby providing insight into the risk of TBW evolving resistance to Bt cotton.

Keywords Cotinine · Gossypol · Alkaloids · ESI/MS/MS · Insect resistance management · Host use

Introduction

Many insect species are highly polyphagous, and many are important economic pests on one or more crop hosts. Understanding patterns of host use is a prerequisite to understanding population dynamics, which, in turn, is necessary for designing appropriate management programs for these pest species. Knowing which host plant species are most responsible for sustaining pest populations and causing pest outbreaks, and when these hosts are used, enables pest monitoring and control programs to be targeted appropriately.

For most important insect pests, the common crop hosts are well-known precisely because that is where these pests cause the most economic impact, but knowledge of weedy and unmanaged hosts is more limited. Furthermore, even where the host range of a pest has been carefully documented, the relative contribution of different hosts to regional metapopulations has rarely been determined because of significant technical barriers. In particular, although larval pest populations are relatively easy to estimate, these populations are highly variable over space and time even on a given host plant species, and thus it is difficult to determine how larval populations on different hosts translate into regional populations.

Armyworms (*Spodoptera* spp.) and heliothines (particularly *Helicoverpa* spp. and *Heliothis* spp.) are important larval pests of cotton, corn, sorghum, and numerous other monocotyledonous and dicotyledonous crops in the Old and New World (Fitt 1989). Adults of these lepidopteran species feed only on nectar and other sugar and moisture sources. To understand the population dynamics of these species, one must determine which plant hosts are the source of the adult moths observed ovipositing in a crop field at a given place and time.

Stable isotope analysis has been used successfully to qualitatively (Gould et al. 2002) and quantitatively (Head et al., unpublished data) infer host use by the important corn, cotton, and vegetable pest *Helicoverpa zea* Boddie. This analysis is based on the principle that the isotopic composition of the wings of adult moths is determined by the physiology of the larval host plant. However, the stable carbon isotope analysis used in these studies could only distinguish whether adults came from larvae that had fed on C3-type hosts (broad-leaved plant species such as cotton, soybean, peanuts, or tobacco) or C4-type hosts (grass-like plant species such as corn, sorghum, or weedy, warm-season grasses). With highly polyphagous pest species like these noctuids, more specific methods are needed to determine the larval host plants from which individual moths originated.

Conceptually, it should be possible to design analyses comparable to stable isotope approaches but focused on plant-derived chemicals that are specific to one or a small set of related plant species. If these analyses are to provide accurate and useful data, the plant-derived analytes need to be preserved in significant (detectable) amounts in adult moths, and they should be unique to the plant species of interest or cause a unique change in the biochemical makeup of the adult moth. Plant secondary metabolites, particularly those involved in plant defense against herbivores, are a promising target for such analyses because many are relatively limited in their taxonomic distribution, and insect herbivores tend to sequester such compounds because of their toxicity.

An example of such a compound is gossypol, which is uniquely related to the lysigenous glands of cotton (*Gossypium* spp.) and closely related species. Work by Rojas et al. (1992) demonstrated that adults of the important cotton pest species, the tobacco budworm (TBW) *Heliothis virescens* (F.), contained gossypol from cotton consumed as larvae. The gossypol was in a bound form; no free gossypol was found. However, the method used pooled samples, and the mass analyzed ranged from hundreds of milligrams to grams.

For an analytical method to be useful for population dynamic and host use studies, it must be suitable for use with large numbers of individual moths, and its selectivity needs to be demonstrated. In addition, having appropriate analyses for several different analytes, each largely or wholly specific to one particular important host plant species, would be particularly useful for gaining an understanding of host use patterns by polyphagous pest species. In the case of *H. virescens*, this would mean having analytical techniques for a cotton-specific compound such as gossypol to determine cotton-derived moths and for a comparable tobacco-specific compound such as nicotine because tobacco (*Nicotiana* spp.) also is a major crop host of this pest.

This paper describes and characterizes novel methods for the analysis of adult moths for both gossypol and cotinine, a metabolite of nicotine. The methods are applied to TBW moths reared on known host plant species and to a set of field-collected TBW moths.

Methods and Materials

Moths For development and characterization of the cotinine and gossypol analyses, TBW larvae were reared individually on leaves of cotton (*Gossypium hirsutum*), tobacco (*Nicotiana spp.*), peanut (*Arachis hypogea*), soybeans (*Glycine max*), and the weedy host, velvetleaf (*Abutilon theophrasti*). Larvae were allowed to pupate, and the resulting adults were freeze-dried for 48 hr. Wings were removed, and the thorax and abdomen of the adults were weighed and frozen for further analysis. The dry weight of the moth bodies ranged from 0.020 to 0.040 g.

Adult male TBW also were collected for cotinine and gossypol analyses adjacent to cotton fields in two North Carolina counties (Lenoir and Wilson) in July and August of 2004. These moths were collected in pheromone traps baited with Zealure (Hercon® Luretape® Insect Attractant Dispenser, Hercon Environmental Company, Emigsville, PA, USA), and pheromone was replaced on a weekly basis. The collected moths were processed as described for laboratory-reared moths.

Chemicals for Cotinine Analysis Chemicals were obtained from the following sources: glacial acetic acid (J.T. Baker ,VWR International, West Chester, PA, USA); ACS-grade methanol (EMD, VWR International); deionized water (Milli-Q Gradient A10 system, Millipore, Billerica, MA, USA); anhydrous diethyl ether (J.T. Baker, VWR International); ethanol (AAPER Alcohol and Chemical Co); 50% solution of sodium hydroxide (J.T. Baker, VWR International). The internal standard d_4 cotinine (98.2% D) was from CDN isotopes (C/D/N ISOTOPES INC., Pointe-Claire, Quebec, Canada), and the cotinine standard from (Sigma-Aldrich, St. Louis, MO, USA).

Chemicals for Gossypol Analysis Chemicals were obtained from the following sources: glacial acetic acid (J.T. Baker,VWR International); gossypol acetic acid standard (95% pure) (Sigma-Aldrich); ACS-grade methanol (EMD, VWR International); HPLC methanol (Burdick & Jackson, Sigma-Aldrich); deionized water (Milli-Q Gradient A10 system

(Millipore); ACS Grade dimethylformamide (DMF) (EMD, VWR International); aniline (99.5% pure) (Sigma-Aldrich); d_5-aniline (98% D) (Sigma-Aldrich); high purity acetonitrile (Burdick & Jackson,WR International); acetone for sample processing (Fisher Certified ACS, Fisher Scientific, Houston, TX, USA); HPLC acetone (Burdick & Jackson, VWR International); ammonium hydroxide (28–30%) (J.T. Baker, VWR International).

Equipment Grinding of moths and extractions were carried out in 96-well polypropylene plates (2.0 ml) (Microliter Analytical Supplies, Suwanee, GA, USA). For grinding, 1- to 6-mm glass balls were used (VWR International). Divinyl benzene (DVB) solid-phase microextraction was carried out by using 96-well SPE Plates (50 or 100 mg DVB, 2 ml capacity), (Supelco [Sigma-Aldrich] and P. J. Cobert, St. Louis, MO, USA). For standards and individual samples, 100-mg DVB 3-ml capacity columns were used (Alltech Associates, Deerfield, IL, USA). The HPLC column was an Agilent Eclipse XDB-C8 (2.1×12.5, 5 μ) guard column. Autosampler 96-well plates (0.5 ml) (Agilent Technologies) were capped with Agilent Technologies 96-well Plate Piercible Cap Mats (P. J. Cobert Associates). The gas chromatograph was an Agilent 6890 (Agilent Technologies, Santa Clara, CA, USA) equipped with a Leap autosampler (Leap Technologies, Carrboro, NC, USA). The detector was a Leco® Pegasus III time of flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA). The capillary column was a 15-m DB5 column with an inside diameter of 0.250 mm and a phase thickness of 0.25 μm (P. J. Cobert Associates).

Analytical Determination of Cotinine To analyze for cotinine and gossypol in the same adult moth requires two different but complementary methods. The extraction of cotinine is carried out first because the gossypol analysis involves a derivatization method. The cotinine determination is accomplished by using d_4 cotinine as an internal standard. The selectivity is achieved by combining a solid-phase extraction (SPE) cleanup procedure with gas chromatography coupled with a mass spectrometer (GC/MS) as the detector . To obtain the requisite selectivity for gossypol, a SPE procedure, followed by electrospray ionization with tandem mass spectrometry, is used. The use of the tandem mass spectrometer gives a high degree of selectivity and minimizes the need for high-resolution, high-pressure liquid chromatography techniques (HPLC). The methods are adapted to a 96-well format, allowing several hundred samples to be processed per day.

The procedure for determination of cotinine was adapted from Kim et al. (2005) and is summarized in Fig. 1a. This involved extraction and a cleanup with a DVB SPE column. Stable isotope-labeled cotinine was added at the beginning of the extraction so that matrix effects could be minimized. The extracted cotinine was concentrated and analyzed directly by GC/MS. The calibration curve for cotinine was obtained by running standards that ranged from 20 to 600 ng. The quantification was carried out by measuring the ratio of the area of *m*/*z* 176 to the area of 180 *m*/*z* where 180 *m*/*z* was the d_4 cotinine internal standard.

To prepare the extraction solution, 50 ml of acetic acid and 200 ml methanol were added to a 1,000-ml volumetric flask, and the volume was brought up to the mark with deionized water. A 20% by volume methanol ether solution was prepared by mixing 20 ml of methanol with 80 ml of ether. A solution of d_4 cotinine was used as an internal standard by dissolving d_4 cotinine in the extraction solution to give a concentration of 100 ng/20 μl.

Individual freeze-dried TBW moths were placed into the wells of a 2-ml 96-well plate. A glass bead was added to each well, and the plate was capped and placed onto a mega grinder and shaken at 1,100 rpm for 45 sec to grind up the moths (Deppermann 2005). One milliliter of cotinine extraction solution, followed by 20 μl of deuterated cotinine internal standard solution, was added to each of the wells. Parafilm was placed over the top of the

plate, and a cap was pressed onto the plate. The moth mixture was vortexed for 15 min, then centrifuged, and the liquid layer removed and added to an 8-ml vial. A second 1 ml of extract solution was added to the moth residue, and the solution was vortexed again for 5 min, centrifuged, and as much liquid removed as possible and combined with the previous extract. One hundred fifty microliters of 40% NaOH, followed by 4 ml of deionized (DI) water, were added to the 8-ml vial containing the extraction solution. The solution was mixed and put through a solid-phase extraction (SPE) DVB column. The 96-well plate containing the moth residue was dried under dry nitrogen and stored at −20°C until needed for the gossypol analysis. An SPE DVB 100 mg (SPE) was used to remove the cotinine from the extraction solution. The DVB solid-phase extraction was prepared by first washing with 2 ml of ethanol, followed by 2 ml of DI water. The cotinine was removed from the extraction solution by passing it through the column. The column was washed with 1 ml of DI water and allowed to dry for 5 to 30 min by drawing or forcing air through the column. The cotinine was eluted with 3.0 ml of 20% methanol/ether. The methanol/ether was removed by using a stream of dry nitrogen. Methanol (150 μl) was added and the sides were carefully washed. The solution was placed into an autosampler vial and injected into a GC/MS.

Gas Chromatography and Mass Spectrometry Conditions for Cotinine The gas chromatography column was a DB-5 column with a length of 15 m, an inner diameter of 0.25 mm, and a film thickness of 0.25 μm. The inlet temperature was 285°C with the injection set for a splitless injection. One microliter was injected. The carrier gas was He, with a flow rate of 2.1 ml/min. The column was held at 100°C for 0.1 min, after which it was ramped to 175°C at a rate of 40°C/min, then to 300°C at 30°C/min. The mass spectrometer was a Leco Pegasus III time of flight. The ionization was through electron impact at 70 eV. The solvent delay was 50 sec. The scan range was from 50 to 210 *m*/*z*. The number of scans was 15 per sec. The ion source temperature was 200°C.

The quantification was carried out by measuring the ratio of the area of *m*/*z* 176 to the area of *m*/*z* 180, where 180 was the d_4 cotinine internal standard. The retention time varied during the running of sets of samples so the retention time was set using the internal standard as a reference. The results for detected cotinine are reported in nanograms.

Analytical Determination of Gossypol The overall method for gossypol analysis of *H. virescens* tissues is summarized in Fig. 1b. The method involved the formation of a Schiff base with aniline, which permits the total amount of gossypol present (bound and unbound) to be determined (Pons et al. 1958; Rojas et al. 1992; Kim et al. 1996). The resulting dianiline complex was further isolated from the moth matrix with divinyl benzene solid-phase extraction, and then was quantified by using high-pressure chromatography coupled with a triple quadrupole mass spectrometer (MS) as the detector. Electrospray ionization (ESI) was used with post addition of ammonia after the LC column. The ESI was operated in negative ionization mode. Because the matrix is complex and the HPLC method was not set for optimum selectivity, the triple quadrupole was operated in reaction monitoring mode. In this mode, the pseudomolecular ion representing the dianiline–gossypol (667 *m*/*z*) was selected by the first quadrupole, followed by collision-induced dissociation in the second quadrupole region. The collision resulted in the loss of one aniline (574 *m*/*z*) and, to a lesser degree, the loss of a second aniline (481 *m*/*z*). These fragments were monitored in the third quadrupole. Deuterated aniline complexed with gossypol was used as an internal standard for the HPLC/MS/MS determination.

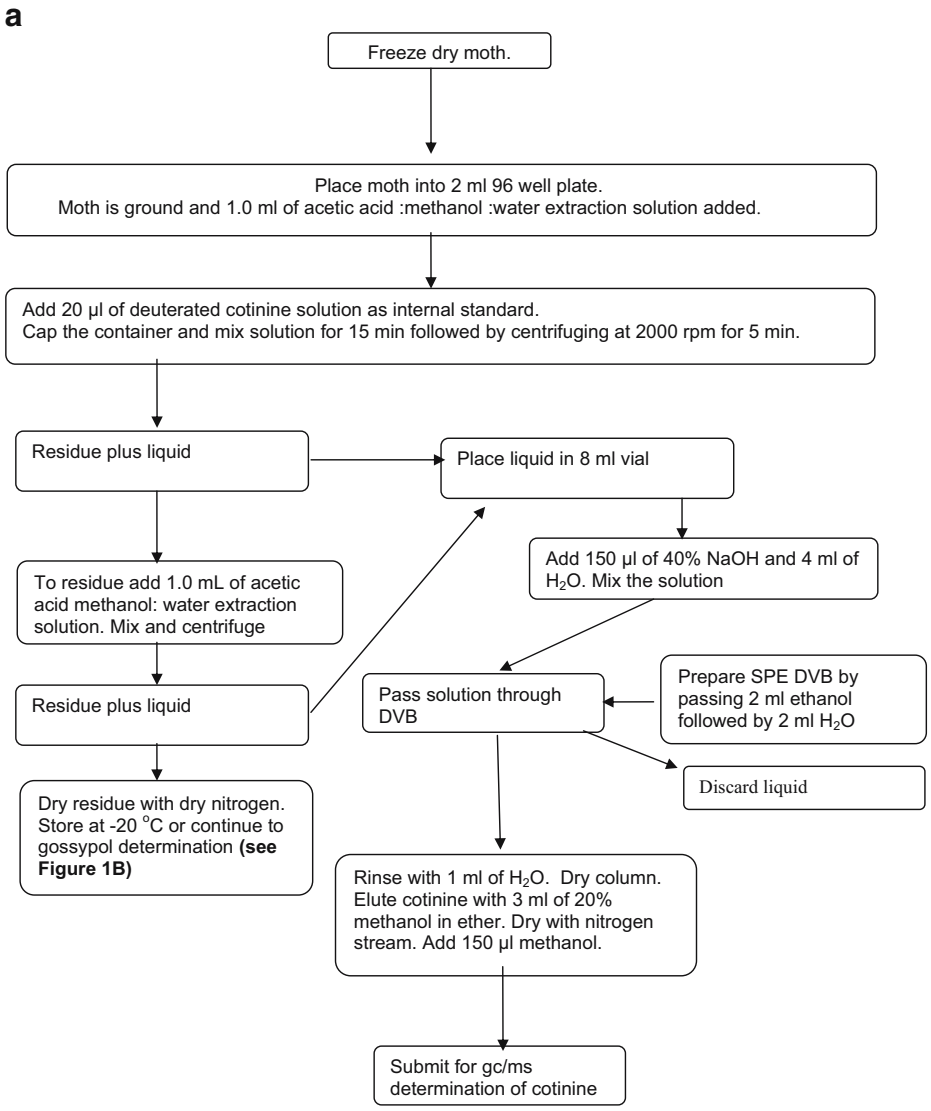


Fig. 1 **(a)** Scheme for cotinine determination in moths. **(b)** Scheme for gossypol determination in moths

The derivatizing reagent was prepared by mixing 1 ml aniline, 5 ml glacial acetic acid, and 44 ml dimethylformamide. This reagent lasted for up to 1 wk if refrigerated at 4°C. The deuterated derivatizing reagent was prepared by mixing 0.1 ml d_5-aniline, 0.5 ml acetic acid, and 4.4 ml dimethylformamide. This was used within 24 hr for preparing the deuterated internal standard. The other necessary solutions were, by volume, 20% water/methanol, 10% water/methanol, 5% acetone/methanol, 50% DMF/water, 0.6% NH_4OH, wash solvent of 100% methanol, acetonitrile/1% acetic acid, and 2:1 acetonitrile/acetone.

The standard stock solution of gossypol was prepared by weighing approximately 10 mg gossypol-acetic acid into a 100-ml volumetric flask, which was filled to the mark with

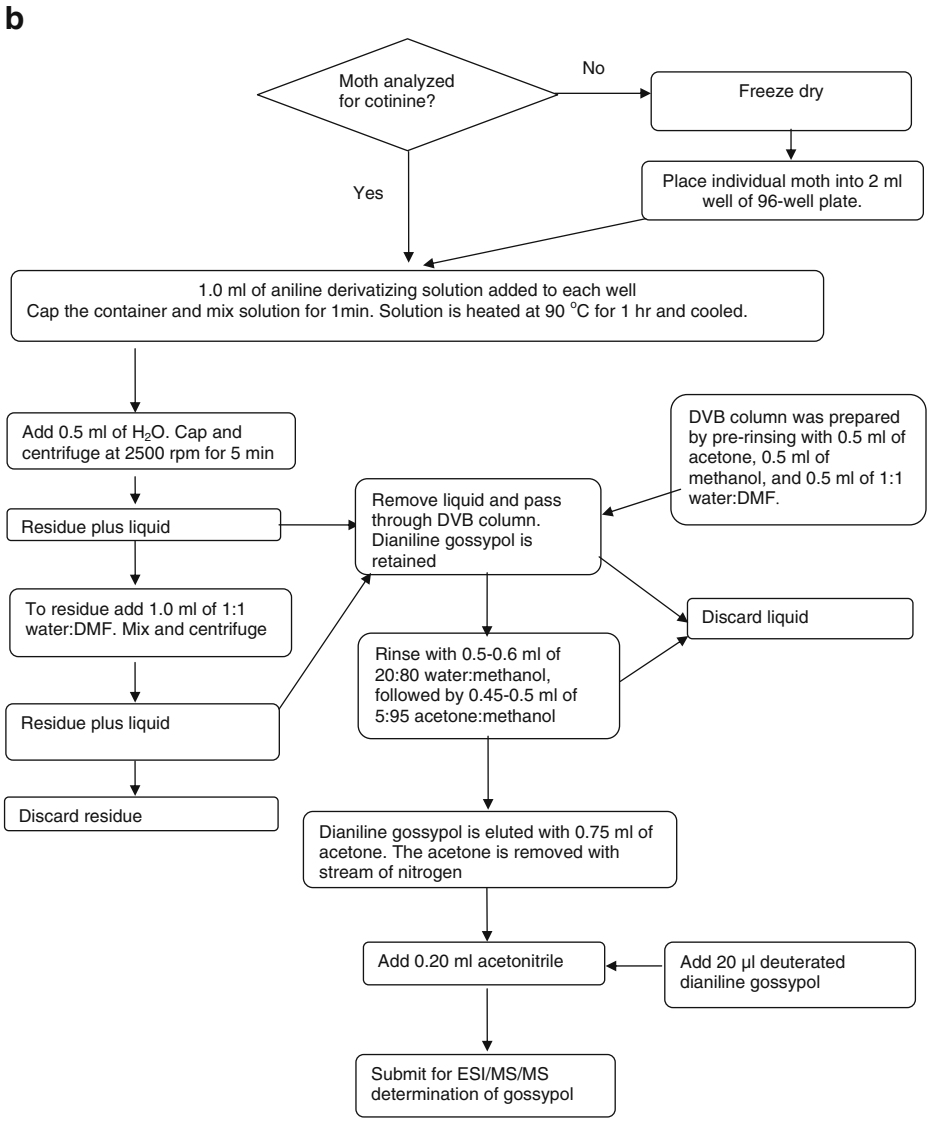


Fig. 1 (Continued)

acetonitrile/1% acetic acid. This solution was stored in an amber glass bottle at 4°C and used within 3 mo. The gossypol-acetic acid standard stock solution was used to prepare a 10 μg/ml standard solution of the dianilino–gossypol by taking 500 μl of the gossypol-acetic acid standard stock solution and removing the solvent under a dry nitrogen stream. To this, 1.0 ml of the aniline derivatizing reagent was added, and the solution was heated at 90°C for 30 min. The excess aniline was removed with a 100 mg DVB SPE column that had been prerinsed with 1.0 ml of acetone, 1.0 ml of methanol, and 1.0 ml of 1:1 water/DMF. The column beds were wetted with the 1:1 water/DMF. One milliliter of DI water was added to the 1.0 ml of dianiline–gossypol solution. This solution was put through the

column, which retained the dianiline–gossypol. The column was rinsed with 0.8 ml of 20:80 water/methanol. The gossypol derivative was eluted from the column with 2 ml of acetone into an amber vial, and the acetone was removed with dry nitrogen. This yielded 50 μg of dianiline–gossypol, which was used with acetonitrile to prepare standards ranging from 25 to 75 ng/ml. These standards were prepared on a daily basis because the dianiline–gossypol was not stable once diluted with acetonitrile.

The deuterated dianiline–gossypol internal standard also was prepared on a daily basis. The 1.0 ml of the standard gossypol stock solution was blown dry, and 1.0 ml of the d_5-aniline derivatizing reagent solution was added to the dry residue. The solution was capped and heated for 30 min at 90°C. To use this d_{10}-dianiline-gossypol as an internal standard, it was necessary to remove the excess d_5-aniline. This was accomplished by using a 100-mg DVB SPE column, which was prerinsed with 1.0 ml of acetone, 1.0 ml of methanol, and 1.0 ml of 1:1 water/DMF. The column beds were wetted with the 1:1 water/DMF, and 1.0 ml of DI water was added to the 1.0 ml of d_{10}-dianiline–gossypol solution. This solution was passed through the column, which retained the d_{10}-dianiline–gossypol. The column was rinsed with 0.5 ml of 20:80 water/methanol, and the d_{10}-dianiline–gossypol was eluted from the column with 2 ml acetone into an amber vial. The acetone was removed with dry nitrogen, yielding 100 μg of d_{10}-dianiline–gossypol. This was used to prepare a 5 μg/ml solution by using acetonitrile.

The individual freeze-dried moths were placed into a 2-ml 96-well plate. For routine analysis, five method blanks, two positive control moths reared on cotton, and two negative control moths reared on plants other than cotton were included. These controls were placed at random in the plate by using a pseudorandom number generator. A glass bead was added to each well, and the plate was capped and placed onto a mega grinder and shaken at 1,100 rpm for 45 sec to grind up the moths (Deppermann 2005). If the moths had been through the cotinine determination, the grinding steps had already occurred. The aniline derivatizing solution (1.0 ml) was added to each well. The plate was sealed by placing a sheet of Parafilm over the top, a new cap was added, and the cap was pressed into place. The plate was vortexed at a low setting for 1 min. The Parafilm and cap were removed, and the plate was covered with aluminum foil and placed in a well heater set at 80–90°C. The solution was heated for 1 hr and then allowed to cool. The samples were cleaned up by using a 96-well DVB plate containing 50 mg of DVB and a positive pressure apparatus (SpeedDisk 96 Pressure Processor from Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA). The 50-mg SPE DVB 2-ml 96-well plate was prerinsed with 0.5 ml of acetone, 0.5 ml of methanol, and 0.5 ml of 1:1 water/DMF. Column beds were wetted with 1:1 water/DMF. Water (0.5 ml) was added to each well of the 96-well plate. The plate was resealed with a clean sheet of Parafilm, capped, and the cap was pressed into place. The plate was centrifuged at 2,500 to 3,000 rpm for 5 min. With a multipipetter, the solution was transferred to the DVB 96-well plate, taking care to leave solid residue behind. The residue was washed with 1.0 ml of 1:1 water/DMF, and this was passed through the DVB 96-well plate. The dianiline–gossypol was retained in the DVB. The DVB column was washed with 0.5–0.6 ml of 20:80 water/methanol, followed by 0.45–0.5 ml of 5:95 acetone/methanol. A clean 96-well plate was placed under the DVB 96-well plate, and the dianiline–gossypol was eluted with 0.75 ml of acetone. The acetone was removed under a dry stream of nitrogen. It was found, at this point, that the samples could be stored for 48 hr without loss of signal. For HPLC/MS/MS determination, 200 μl of acetonitrile were added to each well, along with 20 μl of 5 μg/ml d_{10}-dianiline–gossypol internal standard. The autosampler was equipped for 0.5 ml 96-well plates so it was necessary to transfer the solution to such a plate. The plate was capped and the autosampler was set for less than 18°C.

If only a few samples were to be analyzed, then individual SPE DVB 3-ml columns were used in place of the DVB 96-well plate. The same procedure as for the 96-well DVB plates was followed, except that the volumes were doubled.

HPLC/MS/MS Solvent A was 10% water/methanol and solvent B was acetonitrile/acetone 2:1. The flow rate through the Agilent Eclipse XDB-C8 (2.1×12.5, 5 μ) guard column was set for 0.250 ml/min. The gradient was 0% B for 2.1 min, which then was brought to 60% B in 4 min. Post column, a 0.6% ammonium hydroxide modifier was added at a flow rate of 0.050 ml/min to enhance the negative ion signal. The volume of the sample extract injected was 5 μl. The 0.3 ml/min output of the HPLC system was put directly through the electrospray ionization source of a Micromass Quattro Ultima LC/MS/MS system (Waters, Milford, MA, USA). The electrospray source was operated in negative ion mode. The process forms $M–H^-$ pseudomolecular ions (667 *m*/*z*), which undergo collision activation to form a rearrangement fragment at 574 *m*/*z* reflecting the loss of one aniline. For further confirmation, the loss of a second aniline at 481 *m*/*z* also was monitored. The sensitivity of the loss of the second aniline was relatively low so it was used only for confirmation.

Characterization of the Cotinine and Gossypol Analyses Two methods were used to characterize the accuracy and sensitivity of both analytical methods. First, recovery experiments were conducted with both methods. For the cotinine analysis, cotinine was spiked into samples of TBW adult moths that had not been reared on tobacco. For the gossypol analysis, gossypol was spiked into samples of adult moths that had not been reared on cotton. The spike levels ranged from 5 to 20 ng per moth, and the recovery experiment was replicated over a number of days. The percent recovery was calculated as the ratio of the measured response for the spiked moth to the measured response for the standard. In each case, the response was a ratio of the peak area measured for the analyte to the peak area measured for the internal standard. Because the cotinine method provides an absolute measurement, absolute recoveries could be obtained for the cotinine analysis as compared to relative values for the gossypol analysis.

The second approach for estimating bias was to estimate positive and negative error rates by analyzing adult moths that were reared on known hosts. The gossypol analysis was characterized with a set of 193 TBW moths reared on different known host plants: cotton (118), soybean (7), peanut (5), velvet leaf (30), and an artificial laboratory diet with no cotton content (33). The moths were submitted for analysis with labels that did not indicate on which plant they were reared. Based on the chromatograms for individual moths, Tukey's honestly significantly different (HSD) multiple comparison procedure was used to conduct all possible pair-wise comparisons of the average response ratio and signal-to-noise ratio for the different larval host plants. The statistical analyses were carried out by using SAS Version 8.2 (SAS 2001). A set of criteria for determining if gossypol were present (whether the test was positive) was determined by using a set of cotton reared moths and noncotton reared moths. The criteria were based on the response ratio and the signal-to-noise ratio for the chromatogram signal.

The characterization of the cotinine analysis was carried out in the same fashion but on a smaller scale so that estimates of positive and negative error rates could not be determined. In addition, to determine if the cotinine method and the gossypol method could be used on the same moths, a set of moths reared on different plants were analyzed for both cotinine and gossypol: soybean (3), peanut (3), tobacco (3), and cotton (3).

An additional experiment was conducted to determine if the gossypol signal in adult TBW reared on cotton is sufficiently stable to be detectable under a reasonable range of

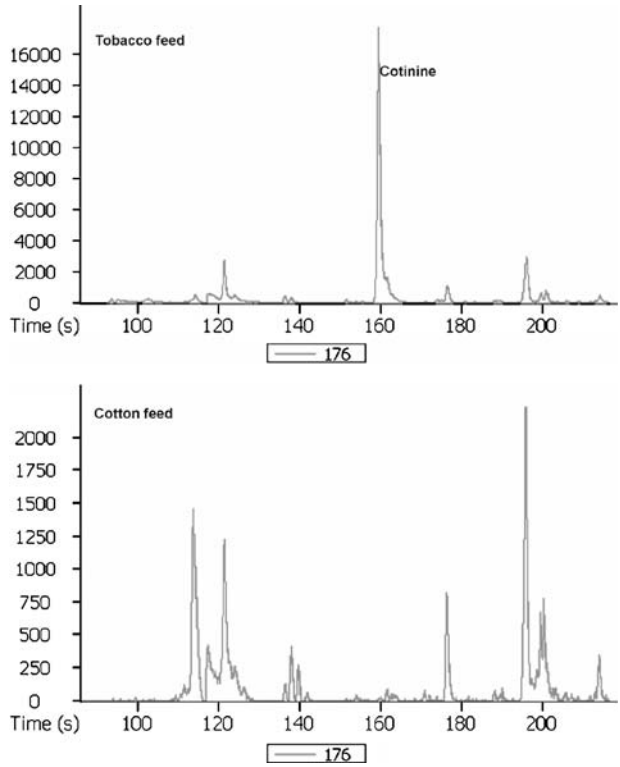


Fig. 2 Ion chromatogram for 176 *m/z* for adult moths reared on tobacco (top) and cotton (bottom). The bottom figure is the ion chromatogram for 176 *m/z* for an adult moth with cotton as the host plant. The peak in the top figure at 159 sec is cotinine. The detection limit represented by the lack of a peak at 159 sec in the bottom figure is 1.5 ppb by dry weight

field conditions. TBW were reared in cages on cotton or chickpea plants in the field. After larvae pupated and emerged as adults, a total of 73 cotton-reared adult TBW were held alive for various lengths of time (0–11 d), then sacrificed and left exposed to the elements for different lengths of time (0–7 d) before being frozen. These treatments were chosen to cover the range of conditions that might occur in collecting moths in the field. Eleven days represents the upper end of the TBW adult lifespan and, therefore, is as long as an adult would be expected to live before being captured in the field. Similarly, moths might remain in a trap for several days after dying and before collection. The 73 cotton-reared moths,

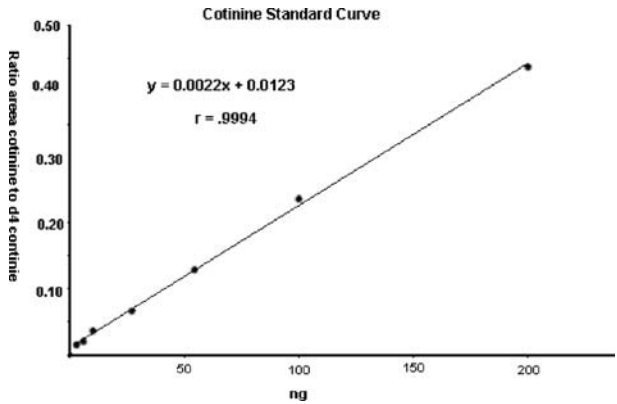


Fig. 3 Calibration curve for cotinine with standards ranging from 2.6 to 200 ng. The detection limit per moth was 0.037 ng

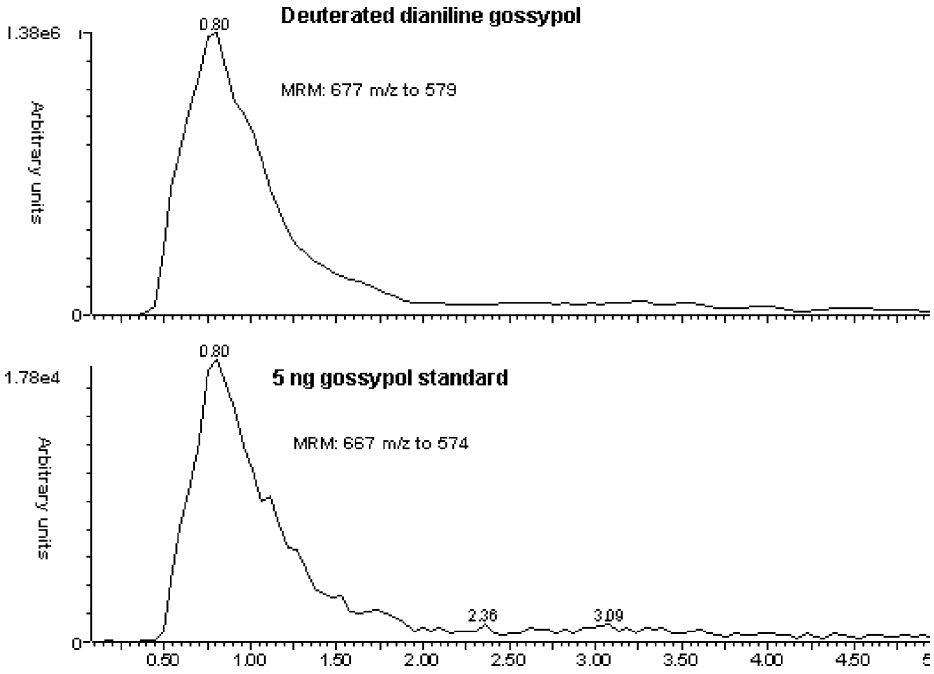


Fig. 4 Reaction monitoring chromatogram for a 5-ng gossypol standard (bottom), and the corresponding chromatogram for a d_{10}-dianiline–gossypol internal standard (top)

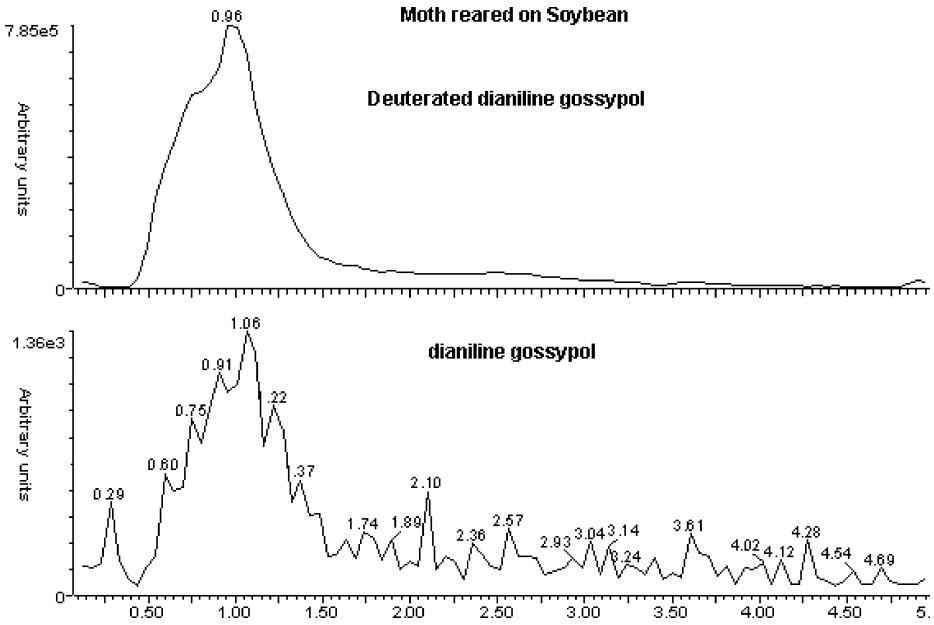


Fig. 5 Reaction monitoring chromatogram for an adult moth reared on soybeans (bottom), and the corresponding chromatogram for an internal d_{10}-dianiline–gossypol standard (top)

together with 19 TBW moths reared on chickpea, were coded and run through a blind analysis for gossypol. Tukey's HSD multiple comparison procedure was used to conduct all possible pair-wise comparisons of the average response ratio and signal-to-noise ratio for the different treatment combinations. The statistical analyses were carried out by using SAS Version 8.2 (SAS 2001).

Application of the Cotinine and Gossypol Analyses to Field-collected TBW Moths To demonstrate the suitability of the cotinine and gossypol analyses for the study of field-collected moths, a small set of moths collected in North Carolina was first analyzed for cotinine and then for gossypol. In total, 30 moths from Lenoir County and 78 moths from Wilson County were analyzed individually as described above.

Results

Cotinine Analysis Figure 2 shows the ion chromatogram for cotinine for a moth reared on cotton and a moth reared on tobacco. The signal for cotinine in the tobacco-reared moth represents 115 ng. The cotinine ion chromatogram for the cotton-reared moth (the bottom chromatogram) represents a detection limit of less than 1.5 ppb by dry weight (Fig. 2). Figure 3 shows the calibration curve for cotinine over the range of 200 to 2.6 ng. The method for cotinine also can provide estimates for nicotine.

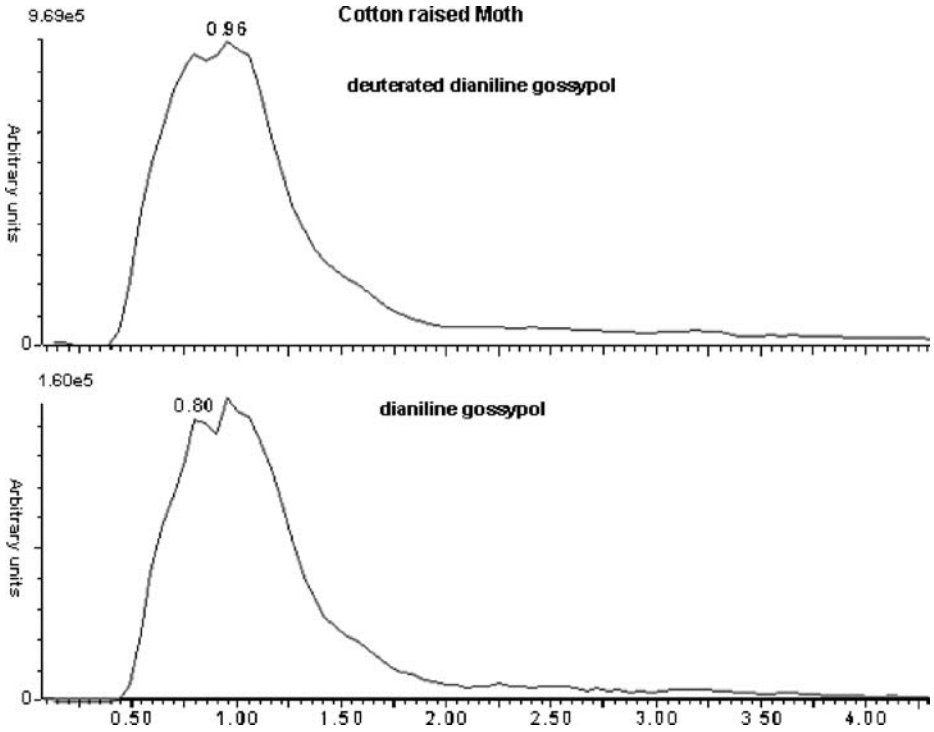


Fig. 6 Reaction monitoring chromatogram for an adult moth reared on cotton (bottom), and the corresponding chromatogram for an internal d_{10}-dianiline–gossypol standard (top)

Table 1 Mean (+ SE) relative response and signal-to-noise ratio from gossypol analyses of TBW moths reared on different hosts, and the percentage of moths judged positive for gossypol

Larval host plant	Cotton (N=118)	Soybean (N=7)	Peanut (N=5)	Velvet leaf (N=30)	Artificial diet (N=33)
Relative response[a]	0.77±0.14a	0.008±0.003ab	0.002±0.002ab	0.03±0.02b	0.01±0.001b
Signal-to-noise ratio[a]	114±8a	8.4±3.6b	0.6±0.6b	6.2±1.2b	9.4±1.1b
Percent positive[b]	100	29	0	13	24

[a] Values in a row followed by the same letter are not significantly different (Tukey's HSD, $P<0.05$).

[b] The following criteria were applied, in order, to determine if a given moth was positive or negative for gossypol: (1) negative if signal to noise ratio <6; (2) positive if response ratio >0.05; (3) negative if response ratio <0.005; (4) positive if signal to noise ratio >10; (5) otherwise negative

Gossypol Analysis Figure 4 shows the reaction ion monitoring chromatogram for a gossypol standard (5 ng in 200 μl). The bottom figure shows the pseudomolecular ion of the dianiline–gossypol 667 *m*/*z* losing an aniline to give 574 *m*/*z*. This represents 125 pg injected. The top portion of Fig. 4 is the corresponding chromatogram for d_{10}-dianiline–gossypol (677 *m*/*z* with loss of one aniline to give 579 *m*/*z*). This represented 2.2 ng that was injected. The chromatograms were normalized to 100. The peak intensity is shown in the upper-right-hand corner and was 1.38×10^6 for the internal standard and 1.78×10^4 for the 5-ng standard. The internal standard was for use with the HPLC/MS/MS system only and did not correct for incomplete conversion to the dianiline–gossypol complex or for recovery.

Figure 5 shows the reaction monitoring chromatogram for an adult moth reared on soybeans; the bottom chromatogram is the response for the dianiline–gossypol and the top is the internal d_{10}-dianiline–gossypol standard as in Fig. 4. The d_{10}-dianiline–gossypol internal standard is not pure and has a small contribution to the dianiline–gossypol signal but the level of d_{10}-dianiline–gossypol was selected so that the contribution would be below detection limits.

Table 2 Results for cotinine analyses followed by gossypol analyses of TBW moths reared on different plant hosts

Larval host	Cotinine[a]	Gossypol[a]
Soy	ND	ND
Soy	ND	ND
Soy	ND	ND
Peanut	ND	ND
Peanut	ND	ND
Peanut	ND	ND
Tobacco	48 ng	ND
Tobacco	21 ng	ND
Tobacco	115 ng	ND
Cotton	ND	+
Cotton	ND	+
Cotton	ND	+

[a] ND, no response detected (for cotinine <1.5 ppb by dry weight and for gossypol based on criteria described in the "Methods and Materials"); +, response detected based on criteria described in Table 1

Table 3 Results of gossypol analysis of TBW moths reared on a noncotton host or on cotton and then sacrificed at various ages and held for various lengths of time after death

Host plant	Moth age (days)	Time in trap after death (days)	Signal-to-noise ratio[a,b]	Relative response[a,b]	Percent positive[c]
Chickpea	NA[d]	NA[d] (*N*=19)	2.7 (1–4) c	0.004 (0.002–0.010) b	0
Cotton	0–5	0-3 (*N*=20)	50 (12–188) b	0.29 (0.024–2.83) a	100
		4-7 (*N*=13)	75 (11–190) ab	0.33 (0.013–1.19) a	100
	6–11	0-3 (*N*=26)	99 (31–228) a	0.58 (0.022–3.48) a	100
		4-7 (*N*=14)	51 (23–139) b	0.18 (0.040–0.49) a	100

[a] Results are expressed as the average signal-to-noise ratio and response for each treatment combination, with the range in parentheses.

[b] Values in a column followed by the same letter are not significantly different (Tukey's HSD, $P<0.05$).

[c] The following criteria were applied, in order, to determine if a given moth was positive or negative for gossypol: (1) negative if signal-to-noise ratio <6; (2) positive if response ratio >0.05; (3) negative if response ratio <0.005; (4) positive if signal-to-noise ratio >10; (5) otherwise negative

[d] NA, not applicable

Figure 6 shows the results for an adult moth that was reared on cotton. The comparison of the adult moth reared on soybean and the cotton-reared adult moth show that the two can be readily distinguished.

Recovery Experiments for Gossypol The recovery experiments were carried out on 16 different days over the course of a 37-d period. The average over all of runs was 111% (SE=10%). If one extreme outlier (240%) is excluded, the average recovery was 102% (SE=5%). The outlier probably resulted from excess aniline remaining in the extraction solution.

Estimation of Positive and Negative Error Rates for Gossypol Method Table 1 shows the results for the analysis of 193 moths reared on known plant hosts. All of the cotton-reared moths were classified as positive for gossypol, indicating that the method has a low rate of false negatives. However, for the criteria used, 14 of the 75 moths reared on noncotton hosts were incorrectly judged as positive for gossypol, indicating that the method yields some false positive results, at least with the decision criteria used in this case. The rate of false positives tended to be higher for moths reared on artificial diet than for moths reared on noncotton plant hosts (25% vs. 16%). However, all of the false positives had relatively low response ratios and signal-to-noise ratios (see Table 2) indicating that it should be possible to adjust the decision criteria to reduce these error rates. This was accomplished in subsequent testing, in which false positive rates were consistently <10% (data not shown).

Table 4 Percentage of TBW moths collected adjacent to cotton fields that were positive for cotinine or gossypol in North Carolina in 2004

County	Month	Number tested	Percent cotinine positive[a]	Percent gossypol positive[a]
Lenoir	July	15	13.3	0
	August	15	6.7	0
Wilson	July	28	46.4	3.6
	August	50	32.0	2.0

[a] Response detection was based on the criteria described in Tables 1 and 2.

Accuracy of Cotinine and Gossypol Analyses When Performed on the Same Insects Table 2 shows the results of cotinine analysis followed by gossypol analysis for a set of moths reared on known plant hosts. All moths reared on tobacco were correctly identified with no errors. Likewise, the moths that were reared on cotton and had gone through the determination for cotinine followed by the gossypol determination were correctly identified as cotton-reared moths.

Stability and Detection of Gossypol in Older and Deceased Moths The results of the gossypol analysis on aged moths are presented in Table 3. The blind analysis correctly identified all moths; 100% of the cotton-reared moths were determined to be positive for gossypol, and none of the moths reared on noncotton plants was determined positive for gossypol. Therefore, a significant gossypol signal was detected in all cotton-reared moths regardless of the age of the moth and/or the length of time the moth was held in a trap after death. The strength of the gossypol signal (measured as either signal-to-noise ratio or as relative response) for cotton-reared moths was always greater than the highest signal observed for moths reared on noncotton plants.

Cotinine and Gossypol Analyses of Field-collected TBW Moths The results of the analyses of the field-collected moths are shown in Table 4. In both counties (Lenoir and Wilson), and in both months (July and August), a higher proportion of the moths was positive for cotinine than for gossypol, indicating that more of the moths developed on tobacco than on cotton. In all cases, the percentage of cotton-derived TBW was less than 4%. The percentage of tobacco-derived moths was higher in the samples from Wilson County than those from Lenoir County, and was higher in the samples from July than in the samples from August.

Discussion

Characteristics and Limitations of the Analyses The prerequisites for the gossypol and cotinine analyses included high selectivity, low detection limits, and the capability to analyze hundreds of samples (insects) per day. The selectivity and high throughput for the gossypol analyses were accomplished by using a combination of analyte isolation followed by the use of tandem mass spectrometry. The gossypol extraction involves the formation of a Schiff base with aniline. The formation of an amine Schiff base is a common approach to determining the total amount of gossypol in a variety of different matrices including cotton (Smith 1967; Stipanovic et al. 1985; Benson et al. 2001), ovine tissues (Kim et al. 1996), blood plasma (Dabrowski et al. 2001), and oils. Bound gossypol is believed to form a Schiff base with proteins (Dabrowski et al. 2001). The addition of excess aniline removes the gossypol from the protein forming an extractable dianilino–gossypol Schiff base.

The sample preparation before determination of gossypol by the HPLC ESI/MS/MS was complicated. When performed by using a single SPE column, no cross-contamination of samples was observed. To accommodate 200 or more analyses per day, the sample processing was adapted to a 96-well plate format. The lyophilized moths were ground with glass beads that could remain in the well and not interfere with the extraction solution. Metal ball bearings were tried initially but interfered with the extraction. Attempts to remove the metal ball bearings caused cross-contamination between wells. Cross-contamination between wells was believed to be the main source of false positives in this approach. Attempts to minimize this included the use of Parafilm between the 96-well cap

and the plate to provide a tight seal during the extraction step. The routine use of blanks and other negative and positive controls were necessary because of the potential for cross-contamination of wells. The blanks were method blanks with no moth present and were placed at random throughout the plate by using a pseudorandom number generator.

As shown in Figs. 4, 5, 6, the dianilino–gossypol detection was complete after 2.5 min. The top of the peak occurred at approximately 1 min. The C8 guard column used in the introduction of the sample to the ESI source helped remove material that diminished the electrospray ionization process, but did not provide the selectivity of a high-resolution analytical column. The best conditions for separation were at low pH. However, these conditions provide poor ionization. The best ionization was observed under neutral or basic conditions. Several other columns were examined with the best separation occurring with a polydivinyl column (PRP-1). However, the solvents used for this column were not conducive to the ESI process. In addition, the time for each analysis was over 12 min. This limited the number of samples that could be analyzed per day. In Figs. 4, 5, 6, the time period after 2.5 min was used for washing the guard column. The total cycle time for each sample injection was 5 min. Thus, over a 20-hr period, 240 samples could be processed through the HPLC ESI/MS/MS system.

The internal standard for the ESI/MS/MS had two main purposes: first, to verify that injection had occurred, and second, to correct for matrix effects that occurred because of the electrospray ionization process. Variation in the mass of the moth caused variation in the electrospray ionization process, leading to similar spike levels producing different peak areas. The lower peak areas appeared to occur with larger moths. The use of the d_{10}-dianiline–gossypol corrected for this variation because the ratio of dianiline–gossypol to d_{10}-dianiline–gossypol remained constant throughout.

The ideal internal standard would be a stable isotope of gossypol. Because this was not available at the time, deuterated aniline was used to generate a stable isotope, which led to some methodological challenges. First, if excess aniline or deuterated aniline remained in solution with the dianiline–gossypol and the d_{10} dianiline, this would lead to exchange of the complexed aniline with the free aniline. If excess aniline were present in the extraction solution, this would have exchanged with the deuterium-labeled aniline of the internal standard, giving rise to aniline-d_5 anilino gossypol and dianiline gossypol, which would have been seen as false positives. If there is no desire to estimate the amount of gossypol present in a sample, the d_{10}-aniline-gossypol could be eliminated and an internal standard for injection such as butylanthraquinone could be used (Benson et al. 2001). This would not correct for matrix effects on the ionization but would provide confirmation of sample injection.

The cotinine determination has all of the components for a highly selective and accurate method. The method uses a stable isotope internal d_4 cotinine standard. The d_4 cotinine is added at the beginning of the extraction procedure for cotinine compensating for recovery losses through all of the steps of the procedure. The high selectivity of the method yields detection limits that are less than 0.037 ng per moth or for a 30-mg moth, a detection limit of less than 1.5 ppb by dry weight. This detection limit is based on a signal-to-noise in the cotinine response of three to one. Therefore, recovery is assumed to be 100% for this level of detection. A nicotine stable isotope can be used in this analysis. However, environmental contamination apparently led to low levels of nicotine being detected in moths reared on host plants other than tobacco. The levels were low compared to moths on which the host was tobacco (data not shown). The likely source of the nicotine contamination was handling of the moths by people using tobacco products.

Applications of the Method One way to slow the rate at which insects evolve resistance to insecticides is to ensure the presence of a refuge where susceptible pest insects are not

exposed to the insecticide. This strategy has been widely used in managing transgenic corn and cotton containing insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (so-called Bt crops). The refuge acts as a source of susceptible pest insects that are available to mate with any resistant insects emerging from the Bt crop fields, thereby "diluting" any resistance present and increasing the durability of the Bt crop.

The refuge may consist of portions of the crop of interest that are untreated, like structured conventional cotton refuges for transgenic Bt cotton. The refuge also may include other suitable crop and weedy hosts of the insect pest (natural or alternative host refuge). Evaluating the refuge available for an insect that is capable of developing on multiple host plant species requires some means of evaluating the portion of the insect population that exists on the different potential hosts. For example, studies of larval host use by the important cotton pest, *Helicoverpa armigera*, in India (Ravi et al. 2005) and China (Wu et al. 2002) demonstrated that many commonly planted crops supported larger larval populations than cotton at the same time that this pest is found feeding on cotton. However, larval surveys of this sort are labor intensive and provide only inferential information on the composition of adult populations. Trapping adults and using suitable diagnostic tools to deduce the host on which a given insect has completed larval development can provide more accurate and comprehensive information about the relative contribution of different plant hosts to regional pest population dynamics.

The method described in this paper has been used for this purpose; the proportion of TBW moths derived from alternative noncotton hosts (the natural refuge) was estimated for populations trapped around Bt cotton fields using the gossypol assay on thousands of individual moths collected in pheromone traps (Head et al., unpublished data). When these assays were run at a large scale, no false negatives were observed with the positive controls, and a rate of <5% of false positives was observed with the negative controls (moths reared on noncotton hosts and method blanks). Therefore, analytical methods that detect the presence of host plant-specific chemicals such as those described in this paper have the ability to quantify accurately host use patterns in polyphagous agricultural pests.

Acknowledgments We thank Ryan Jackson (USDA-ARS, Stoneville, MS) for providing the TBW moths reared on known hosts and for coding samples for the blind experiment on gossypol signal stability. James Jennings, Melinda McCann, and Eric Sachs (Monsanto Company) provided helpful comments on the manuscript. Sharon Alexander and Mary Anderson (Monsanto Company) contributed tireless technical assistance with the gossypol assays.

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J Chem Ecol (2007) 33:1149–1159
DOI 10.1007/s10886-007-9282-5

Isolation, Identification, and Quantification of Potential Defensive Compounds in the Viceroy Butterfly and its Larval Host–Plant, Carolina Willow

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Received: 4 October 2006 / Revised: 26 January 2007 / Accepted: 27 February 2007 /
Published online: 13 April 2007
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Abstract The viceroy–monarch and viceroy–queen butterfly associations are classic examples of mimicry. These relationships were originally classified as Batesian, or parasitic, but were later reclassified as Müllerian, or mutalistic, based on predator bioassays. The Müllerian reclassification implies that viceroy is unpalatable because it too is chemically defended like the queen and the monarch. However, unlike the queen and the monarch, the viceroy defensive chemistry has remained uncharacterized. We demonstrate that the viceroy butterfly (*Limenitis archippus*, Nymphalidae) not only sequesters nonvolatile defensive compounds from its larval host–plant, the Carolina willow (*Salix caroliniana*, Salicaceae), but also secretes volatile defensive compounds when disturbed. We developed liquid chromatography–mass spectrometry–mass spectrometry methods to identify a set of phenolic glycosides shared between the adult viceroy butterfly and the Carolina willow, and solid phase microextraction and gas chromatography–mass spectrometry methods to identify volatile phenolic compounds released from stressed viceroy butterflies. In both approaches, all structures were characterized based on their mass spectral fragmentation patterns and confirmed with authentic standards. The phenolics we found are known to deter predator attack in other prey systems, including other willow-feeding insect species. Because these compounds have a generalized defensive function at

Electronic supplementary material The online version of this article (doi:10.1007/s10886-007-9282-5) contains supplementary material, which is available to authorized users.

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the concentrations we described, our results are consistent with the Müllerian reclassification put forth by other researchers based on bioassay results. It seems that the viceroy butterfly possesses chemical defenses different from its monarch and queen butterfly counterparts (phenolic glycosides vs. cardiac glycosides, respectively), an unusual phenomenon in mimicry warranting future study.

Keywords Chemical defense · Chemical mimicry · *Limenitis archippus* · LC/MS/MS · Nymphalidae · *Salix caroliniana* · Salicaceae · SPME-GC/MS

Introduction

Visual defensive mimicry, defined as close physical resemblance between unrelated species (Bates 1862), is an enduring example of adaptation by natural selection (Fisher 1958; Charlesworth and Charlesworth 1975; Ruxton et al. 2004; Thompson 2005). Mimicry is generally classified as either Batesian or Müllerian. In Batesian mimicry, a rewarding (i.e., palatable) species evolves a physical resemblance to the warning phenotype of a nonrewarding (i.e., unpalatable) species. This relationship is considered parasitic because the palatable species undermines the effectiveness of the warning signal. In contrast, Müllerian mimicry involves two unpalatable species with both participants sharing a common physical appearance. In a Müllerian system, both species benefit by distributing the mortality costs of predator education, and thus the relationship is generally considered mutualistic (Mallet 1999). Therefore, correctly classifying the mimicry system has important ramifications regarding the ecology and evolution of the involved participants.

The monarch (*Danaus plexippus*) and queen (*Danaus gilippus*) butterflies (Nymphalidae: Danainae) arrived at the forefront of biological interest as Batesian models of the viceroy butterfly (*Limenitis archippus*) (Nymphalidae: Limenitinae) based on predator bioassays (Brower 1958a, b). In these behavioral experiments, monarch and queen butterflies were more unpalatable than viceroy butterflies to avian predators. Furthermore, monarch and queen larvae feed on milkweeds (Asclepiadaceae), and both insect species are able to sequester and retain bitter and toxic cardenolides present in milkweeds into their adult stage (reviewed in Ackery and Vane-Wright 1984; Brower et al. 1984). Cardenolides are known to contribute to prey unpalatability, resulting in avoidance learning by both vertebrate (Glendinning 1992) and invertebrate (Berenbaum and Miliczky 1984; Prudic et al. 2007) predators. Conversely, viceroys were originally shown to be relatively palatable (Brower 1958a, b), and were not known to sequester noxious compounds from their larval host–plants, willows and poplars (Nishida 2002). However, subsequent behavioral bioassays led to a re-evaluation of these relationships. In those assays, viceroys were equally or more unpalatable to avian predators than their monarch and queen counterparts, and this shared unpalatability increased the rate of predator aversion learning (Ritland and Brower 1991). These results led the authors to conclude that viceroy butterflies were Müllerian, not Batesian, mimics with queens and monarchs.

Ritland and Brower's conclusions imply that viceroy butterflies, like monarch and queen butterflies, are protected by chemical compounds that render them unpalatable to predators; however, these putative defensive compounds were not known. This absence of knowledge about underlying defensive mechanism provoked criticisms of the predator bioassay results and interpretations (Guilford 1991). In light of these criticisms, a chemical explanation for viceroy unpalatability would go far in deciding whether viceroys are Müllerian or Batesian mimics and understanding their ecological interactions and evolutionary trajectories. If the

viceroy is a Batesian mimic, it should not possess chemical defenses. However, if the viceroy is a Müllerian mimic, then it should have chemical defenses (Rothschild 1991). We sought to determine if the viceroy was indeed chemically defended and is thus consistent with the Müllerian classification.

A critical first step in such chemical characterization was determining candidate compounds associated with unpalatability. Many different chemical classes contribute to the unpalatability of Lepidoptera, including aristolochic acids, cardenolides, cyanogenic glycosides, iridoid glycosides, and pyrrolizidine alkaloids (Nishida 2002). Although they have not previously been identified in any butterfly species, we considered phenolic glycosides and their chemical relatives as the most likely candidate compounds for viceroy butterfly unpalatability because (1) these compounds are ubiquitous antiherbivore compounds of the Salicaceae (willows and poplars), the larval host–plants of the viceroy butterfly (Palo 1984; Tahvanainen et al. 1985; Lindroth et al. 1988b); (2) these compounds are sequestered by other insects of the willow-feeding guild, such as the beetles *Phratora vitellinae* and *Chrysomela* spp. (Coleoptera: Chrysomelidae) (Pasteels et al. 1988; Köpf et al. 1998); and (3) these compounds when present in insects deter attack by generalist predators (Pasteels et al. 1986; Rank et al. 1996; Müller et al. 2006). By using high-performance liquid chromatography–mass spectrometry (LC/MS), and gas chromatography–mass spectrometry (GC/MS), we investigated whether (1) viceroy butterflies sequester nonvolatile defensive phenolic glycosides from their larval host–plants; (2) viceroy butterflies emit volatile defensive phenolic compounds when disturbed; and (3) both the identity and the concentrations of the compounds we found have been shown to be predator deterrents in other bioassay experiments.

Methods and Materials

Natural History

The viceroy butterfly is a member of *Limenitis* (Nymphalidae, Limenitidinae). Three of the four members of this genus in North America are involved in some type of mimicry relationship with vastly different butterfly species (Prudic et al. 2002 and references therein). According to phylogenetic analysis, the ancestral phenotype for all North American *Limenitis* is a black ground color with a white dorsal band (Mullen 2006). The viceroy adult phenotype is diverged from this ancestral pattern with an orange or brown ground color with black markings. Viceroy butterflies (*Limenitis archippus*) are widely distributed over North America, and they exhibit regional variation in adult color pattern (Scott 1986) that correlates with geographic distribution of their models (Ritland and Brower 2002). In areas where the monarch is prevalent, the viceroy exhibits a tawny orange ground color with black markings (*Limenitis archippus archippus, Limenitis archippus lahontani*), like the monarch. Conversely, viceroy butterflies in areas where the queen butterfly is more frequent exhibit a color pattern similar to the queen, which is a more brownish-orange ground color with black markings (*Limenitis archippus floridensis* and *Limenitis archippus obsoleta*). Viceroy larvae primarily feed on *Salix* and *Populus* (Salicaceae), unlike monarch and queen larvae that feed on members of the Asclepiadaceae (Scott 1986). In Florida, where the biological collections for this study were done, the viceroy butterfly (*L. archippus floridensis*) is believed to mimic the queen, not the monarch butterfly, and the viceroy larvae feed almost exclusively on the Carolina willow, *Salix caroliniana* (Ritland and Brower 1991).

Butterfly and Host–Plant Collections

Adult viceroy butterflies and their larval host–plants were collected between July 20–21, 2004 from two locations in Florida, USA: Green Meadow Road, Lehigh Acres, Lee County, N26°31.69′, W−81°40.67′ and Newnan's Lake, Gainesville, Alachua County, N29°38.19′, W−82°12.00′. These sites are close to the viceroy collection sites used in previous predator bioassays (Brower 1958b; Ritland and Brower 1991). For the LC/MS analysis, four samples each of butterflies and willow were collected from both locations. A butterfly sample consisted of 10 adults (either all male or all female) (~1 g dry weight), whereas a willow sample consisted of 16 young leaves, 2 leaves from 8 different plants (~10 g dry weight). All samples were weighed, then air-dried at room temperature for 1 wk, and re-weighed. For the GC/MS analysis, live butterflies were individually sampled by using both solid phase microextraction (SPME) and direct sampling of the putative defensive secretion in the laboratory. Butterflies were caught in the field and analyzed the next day before being fed. There were five males and five females per site, and each individual butterfly secretion was analyzed separately.

Authentic Standards

Benzoic acid, benzaldehyde, salicylaldehyde, and salicin standards were purchased from Sigma Chemical Co. (USA). A salicortin standard was provided by C. Orians (Tufts University, USA), and a tremulacin standard was purchased from Apin Chemical Ltd. (Abingdon, Oxon, UK).

High-performance Liquid Chromatography–Mass Spectrometry

Sample Extraction and Preparation The Green Meadow Road samples had the following dry weight: willow 1, 11.11 g; willow 2, 10.90 g; male viceroy 1, 0.89 g; and female viceroy 1, 0.91 g. The Newnan's Lake samples weighed the following: willow 3, 9.24 g; willow 4, 10.88 g; male viceroy 2, 0.99 g; and female viceroy 2, 1.17 g. Whole butterflies including their wings were used in the extraction. Dried samples were ground to a fine powder before methanol extraction. Methanol (50 ml viceroy, 200 ml willow) was added to each sample, sonicated for 30 min, and then allowed to soak overnight. The mixture was partitioned with an equal volume of hexane, ×3. The methanol fraction was concentrated to dryness, weighed, and 1 mg samples were resuspended in 1 ml of high-performance liquid chromatography (HPLC) grade methanol, then filtered through a 0.45-μm PTFE filter (Whatman) for analysis.

Identification of Nonvolatile Defensive Compounds Chromatograms were obtained by using an Agilent 1100 HPLC system (quaternary gradient pump, diode array detector, thermostated column compartment, and autosampler) with a 4-μm Waters Nova Pack Sentry guard column (3.9×20 mm) and Waters Nova Pak reverse phase C18 column (4.6×150 mm). Each sample (20 μl) was injected into a methanol–water gradient flowing at 1 ml/min (N=8) (solvent gradient described in Table 1). UV signals were observed at 200, 230, 277, and 300 nm. For LC/MS, analyses of each sample were obtained with an Agilent 1100 HPLC system tandem with Agilent MSD-Trap-SL ion trap mass spectrometer. LC parameters were the same as above. The MS acquisition parameters were positive electrospray ionization mode, drying gas temperature 350°C, drying gas flow rate 10 l/min, nebulizer pressure 35 psi, HV capillary 3500 V, HV end plate offset −500 V, capillary current 24.4 nA, current end plate 1,138.6 nA, RF amplitude capillary exit 158.5 V, and skimmer 40.0 V.

Table 1 LC solvent gradient with flow rate of 1.00 ml/min

Time (min)	% Water+0.05% Acetic Acid	% Methanol+0.05% Acetic Acid
0.00	100	0
3.50	80	20
7.00	55	45
15.00	50	50
19.00	5	95
20.00	0	100
26.00	0	100
29.00	85	15

Compounds were identified by comparing their retention times and mass spectra (MS and MS/MS) with those of authentic standards.

Quantification of Nonvolatile Defensive Compounds Quantification was done by external standard method using a six-point standard curve with standards ranging from 0 to 2.0 mg/ml. Calibration curves were constructed for the three phenolic compounds by using LC/MS protocol described above. For each compound, a characteristic product ion was chosen from its MS/MS as its quantification ion (Table 2). Peak integration and quantification were performed automatically with Agilent ChemStation software (version A.10.01). The same samples used in identification analyses were re-run twice for quantification to ensure consistency within a sample. Concentrations were considered consistent if run 1 and run 2 were within 10% of each other. If not, then the sample was reinjected until the two runs reached the consistency criteria. However, only the concentration of the first run was used for reporting and statistical analyses (N=4 willow samples; N=4 viceroy samples). All statistical analyses were performed in JMP-IN software (SAS Institute Inc. 2002) with one-way ANOVAs.

Gas Chromatography–Mass Spectrometry

Identification of Defensive Volatiles The viceroy butterfly secretes a malodorous fluid from its abdomen when disturbed. To characterize the volatile compounds that a predator would encounter when it disturbs a viceroy, StableFlex PDMS/DVB-coated SPME fibers (Supelco, USA) were used to sample the volatiles in the headspace above the abdomen

Table 2 Compound-specific LC/MS and LC/MS/MS identification and quantification results of viceroy butterfly and its larval host–plant

Compound	Retention Time (min)	Diagnostic Ions (m/z) Parent/Daughters	Willow Conc.±SD (mg/g dry wt.) (N=4)	Viceroy Conc.±SD (mg/g dry wt.) (N=4)
Salicin	6.8	309:185,[a] 277	3.55±0.91	3.04±2.02
Salicortin	8.9	442:263,[a] 268, 245	25.36±2.93	6.56±3.74
Tremulacin	18.6	546:267,[a] 373	34.90±3.54	26.69±15.91

For salicin the extracted ion was 309 m/z [M+Na] from 5.0–7.0 min; for salicortin the extracted ion was 442 m/z [M+H_2O] from 8.0–10 min; and for tremulacin 546 m/z [M+H_2O] from 18.0–20.0 min.

LC–MS–MS = liquid chromatography–mass spectrometry–mass spectrometry

[a] Quantification ion

of both stressed and normal butterflies (Borg-Karlson and Mozuraitis 1996). To simulate a predatory event, a butterfly was pinned horizontally by the wings between a glass plate and a glass cylinder with one small opening (400 ml beaker) (Supplemental Material Fig. 1). This arrangement positioned the butterfly legs parallel to the glass plate without any surface for the butterfly to perch on. A SPME fiber holder with an averted fiber was placed in the small opening for 1 hr of sampling of the area directly above the struggling butterfly and its secretion. To collect the volatile compounds emitted by an unstressed butterfly, the same individuals were placed in the same container and sampled as described; however, the butterfly was not pinned down to simulate a predation event. The butterfly was able to wander, or more often perch, on the glass plate. Separate SPME fibers were used for stressed and normal sampling events. The glassware was cleaned between sampling bouts with methanol. Each butterfly was sampled twice for each treatment to ensure consistency of the results (N=20).

Data were collected with a Varian Saturn 2100T GC/MS and a Chrompack capillary column (CP Sil 8 CB; 30 m×0.25 mm; 0.5 μm film thickness). The initial column oven temperature was set for 80°C, increasing to 210°C at 10°C/min; the injector, transfer line, and trap temperatures were 230, 250, 200°C, respectively, and the electron voltage was set at 70 eV. Ultra high purity helium was used as the carrier gas at a flow rate of 1.0 ml/min. Each volatile sample was injected directly into the chromatograph after desorbing from the SPME fiber for 15 sec in the GC injector (230°C). Compounds were identified by comparison of retention times and mass spectra to authentic standards and the NIST 02 MS library.

Quantification of Defensive Volatiles Volatile compounds were quantified by the external standard method using a six-point standard curve with standards ranging from 0.005–5.0 μl/ml. Calibration curves from triplicate injections of 2.0 μl were obtained by using the GC/MS protocol above. Peak integration and quantification were performed automatically with Saturn 2100 Workstation software. The same insects used for compound identification were reanalyzed for compound quantification; however, for this analysis, the secretion was sampled directly from the abdomen of the butterfly with a glass capillary (Supplemental Material Fig. 2). Two microliters of the secretion were collected from a disturbed butterfly and dissolved in 2.0 μl of ethyl acetate with 1.0 μl of 0.25 M *p*-chlorotoluene as the internal standard. Then, 2.0 μl of this solution were injected directly into the GC column. Each butterfly sample was run twice on the GC for consistency. The concentrations were considered consistent if run 1 and run 2 were within 5% of each other. If not, then the individual butterfly was resampled until the two runs reached the consistency criteria. However, only the concentration of the first run was used for reporting and statistical analyses (N=20). All statistical analyses were performed in JMP-IN software (SAS Institute Inc. 2002) using one-way ANOVAs.

Results

In the LC/MS assays, we characterized the tissue chemical profiles of the Florida viceroy (*L. archippus floridensis*) and its larval host–plant Carolina willow (*S. caroliniana*). Of the five peaks shared between the viceroy and willow extracts, three corresponded to phenolic glycosides known to be unpalatable to either herbivores or their predators: salicin, salicortin, and tremulacin (Fig. 1; Table 2). These compounds were found in both the willows and butterflies across sites and between sexes. Geographic location and butterfly sex did not significantly affect the concentration levels of any of the three phenolic glycosides (one-way ANOVAs, P>0.05) (Table 3).

In the GC/MS assays, we characterized and quantified the volatile chemical profile of a viceroy during a predation simulation and compared it to the volatile profile of a viceroy at rest. During these collection bouts, an unstressed viceroy never emitted a secretion, but the stressed butterflies always did. By using SPME in the stressed butterfly, we identified four compounds not found in the unstressed butterfly: benzaldehyde, benzoic acid, methyl salicylate, and salicylaldehyde (Table 3). These were identified both in the headspace surrounding the provoked butterfly and in its secretion. Again, all four compounds were found in butterflies across geographic location and between butterfly sexes. There were significant differences in geographic concentrations in benzaldehyde ($F_{1,19}$=10.24, P=0.001), benzoic acid ($F_{1,19}$=12.35, P<0.001), and salicylaldehyde ($F_{1,19}$=11.91, P<0.001) (Table 3). Butterflies from the northern Florida site, Newnan's Lake, had higher concentrations than butterflies from the southern Florida, Green Meadow Road.

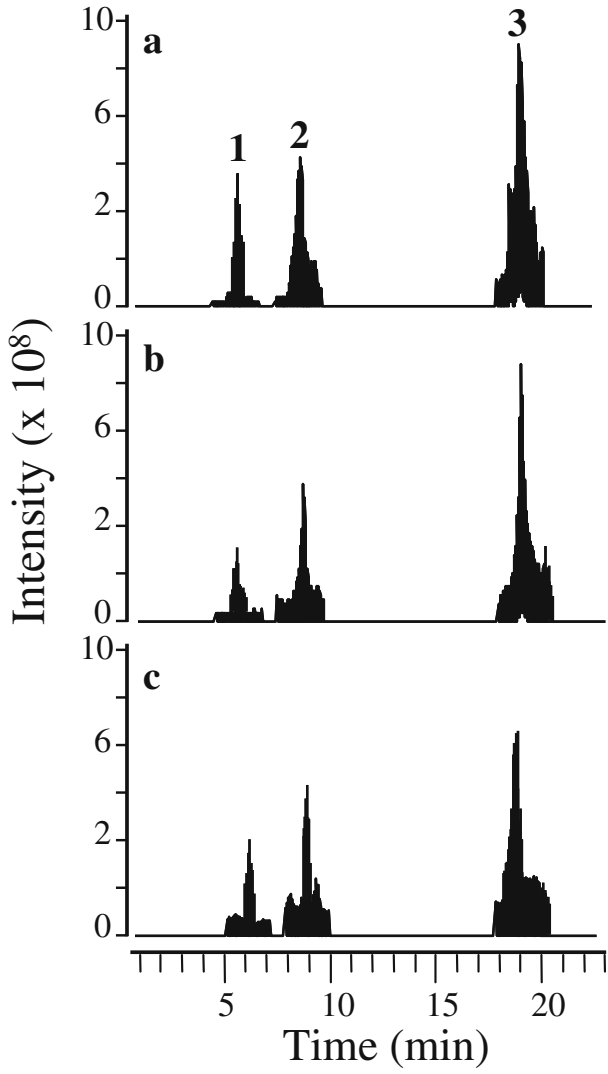


Fig. 1 Extracted ion chromatograms of shared phenolic glycosides of Carolina willow and viceroy butterfly compared to authentic standard mixture. **(a)** Standard solution containing three phenolic glycosides: salicin (peak 1), salicortin (peak 2), and tremulacin (peak 3). **(b)** Carolina willow sample 1 from Green Meadow Road site. **(c)** Adult viceroy butterfly sample 1 (10 males) from Green Meadow Road site. Specific site information is provided in the methods and materials section

Table 3 Concentration averages of nonvolatile phenolic glycosides in willow and viceroy butterfly (total, geographic location, and sex) (LC/MS) and concentration averages of volatile phenolics released from stressed butterflies (total, geographic location, and sex) (GC/MS)

Compound	Willow Conc.±SD		Viceroy Conc.±SD		
	Total Conc.	Location	Total Conc.	Location	Sex
Benzaldehyde[a]	NS	NS	4.31+2.56	2.17+0.82 (GMR) vs. 6.45+1.73 (NL)[b]	3.42+2.10 (M) vs. 5.21+2.74 (F)
Benzoic acid[a]	NS	NS	0.14+0.13	0.03+0.01 (GMR) vs. 0.25+0.09 (NL)[b]	0.15+0.13 (M) vs. 0.13+0.11 (F)
Methyl salicylate[a]	NS	NS	28.01+10.60	27.64+2.24 (GMR) vs. 28.33+10.11 (NL)	26.48+10.39 (M) vs. 29.54+11.03 (F)
Salicin[c]	3.55+ 0.91	3.19+0.88 (GMR) vs. 3.90+1.10 (NL)	3.04+2.02	2.12+1.29 (GMR) vs. 4.97+1.12 (NL)	2.55+2.30 (M) vs. 3.55+3.14 (F)
Salicortin[c]	25.36+ 2.93	24.11+2.38 (GMR) vs. 26.61+3.72 (NL)	6.56+3.74	6.52+3.83 (GMR) vs. 9.75+1.31 (NL)	5.67+4.52 (M) vs. 7.45+4.57 (F)
Salicylaldehyde[a]	NS	NS	6.12+4.89	0.82+0.01 (GMR) vs. 9.99+4.04 (NL)[b]	6.86+6.18 (M) vs. 5.38+3.26 (F)
Tremulacin[c]	34.90+ 3.54	36.5+3.37 (GMR) vs. 33.35+4.00 (NL)	26.69+15.91	12.94+2.58 (GMR) vs. 40.18+3.37 (NL)[b]	24.46+18.83 (M) vs. 28.67+19.60 (F)

[a] Measured in micrograms per microliter (total, N=20; geography, N=10; sex, N=10)

[b] Significant difference (one-way ANOVA, $P<0.05$)

[c] Measured in micrograms per gram dry weight (total, N=4; geography, N=2; sex, N=2)

F = Female, GMR = Green Meadow Road, M = male, NL = Newnan's Lake, NS = not sampled

Discussion

Originally, the viceroy butterfly was widely regarded as a palatable, Batesian mimic without any form of chemical defense (Brower 1958a, b). However, subsequent predator bioassays suggest that it is unpalatable and chemically defended against predation. These results led to a re-classification of the system as Müllerian (Ritland and Brower 1991). Our results are consistent with the Müllerian, not the Batesian, classification. We have shown that adult viceroys possess compounds that function as defensive compounds in other willow-feeding insects. We found three phenolic glycosides (salicin, salicortin, and tremulacin) in the larval host–plant, the Carolina willow, and the adult viceroy (Fig. 1; Table 2). It is known that salicin can be a sample preparation artifact; however, our sample preparation protocol limited this possible bias (Julkunen-Tiitto and Sorsa 2001). All three phenolic glycosides were found in the butterflies and the willows from both populations and in both sexes of butterflies, but there were no statistical differences in compound concentration between either geographic location or butterfly sex (Table 3). We also found the viceroy emitted four volatile compounds (benzaldehyde, benzoic acid, methyl salicylate, and salicylaldehyde) when disturbed in a simulated predation event. All these compounds were found in

butterflies from both locations and in both sexes, although concentrations of benzaldehyde, benzoic acid, and salicylaldehyde were statistically higher in butterflies from northern Florida compared to butterflies from southern Florida (Table 3). Future sampling that focuses on more populations is needed to verify these preliminary patterns of geography and sex on viceroy chemical defense profiles and concentrations.

This is the first time that Carolina willow (*S. caroliniana*) has been chemically characterized for phenolic glycosides, and the three phenolic glycosides we documented resemble the chemical profiles of other willow species (Palo 1984; Nyman and Julkunen-Tiitto 2005) and other members of the Salicaceae in general (Lindroth et al. 1988a; Lindroth and Hemming 1990). Typically, when tremulacin is found in willows, it is accompanied by salicortin and salicin (Julkunen-Tiitto 1989). The Carolina willow had average levels of salicin, salicortin, and tremulacin compared to other *Salix* spp. (Soetens et al. 1998), and these quantities have been shown to deter generalist herbivores (Palo 1984; Tahvanainen et al. 1985).

This is also the first example of adult butterflies containing phenolic glycosides (compare with Nishida 2002). In this case, they are probably derived via sequestration from the larval host–plant. Although our study did not address if these compounds deter predation on viceroys, the results from other bioassay experiments suggest this is a likely function. Willow-derived phenolic glycosides, including the compounds we found, are known for their deterrent and toxic effects on a variety of insect predators (reviewed in Bairlein 1997). Salicin, when present in leaf beetles at concentrations similar to those found in the viceroy butterflies, deters predators (Pasteels et al. 1986). Salicortin and tremulacin have been evaluated together, not individually, for their defensive properties against predators. When these two compounds are present in gypsy moth caterpillars at concentrations similar to the viceroy, they are deterrent and toxic, and they also negatively affect diet choice in insectivorous songbirds (Müller et al. 2006).

The volatile compounds, benzaldehyde, benzoic acid, and salicylaldehyde, are well-documented chemical defenses of willow-feeding leaf beetles against a variety of generalist predators (Smiley et al. 1985; Pasteels et al. 1988; Denno et al. 1990). The concentrations of these that we documented were lower than in some other studies; however, they are within the range of concentrations that are effective in conferring resistance to predation (Pasteels et al. 1988; Denno et al. 1990). Methyl salicylate (wintergreen oil) has not been evaluated as a deterrent to avian or invertebrate predators. However, it is described as the most poisonous of the salicylates; doses less than 1 g have killed small children weighing 10 kg (Michael and Sztajnkrycer 2004). Concentrations as low as 2 mg/ml also induce nausea and vomiting when ingested by mice (Davidson et al. 1961). It is not presently known how viceroys acquire these volatile compounds. Some are known to occur in the foliar scent of willows (Dötterl et al. 2005), and thus may be sequestered from the larval host plant or acquired through adult nectar feeding. Regardless of the exact mechanism, our data demonstrate that viceroy butterflies contain known toxic and deterrent phenolic compounds.

Our chemical results are consistent with other researchers' reclassification of the viceroy–queen and viceroy–monarch butterfly mimicry as Müllerian. Viceroys likely share the costs of predator education with their co-mimics. Unlike other Müllerian mimicry systems such as the one involving *Heliconius* butterflies, the shared appearance and unpalatability between the viceroy and the monarch and queen did not arise through either common ancestry or shared host–plant use. Thus, a comparison of these two types of Müllerian systems will provide a framework for understanding the relative roles of host–plant use, sequestration, and genetic architecture of wing coloration in Müllerian mimicry and aposematic coloration. This system is also one of the few Müllerian examples where

the participants unmistakably vary in defensive chemistry: the adult viceroy sequesters defensive phenolic glycosides from its larval host–plant, whereas the queen and monarch butterflies sequester defensive cardiac glycosides from their larval host–plant (Brower et al. 1984). Although these compound classes differ structurally and functionally, recent experimental evidence indicates that such differences could actually facilitate Müllerian mimicry evolution by increasing rates of predator aversion learning and remembering (Skelhorn and Rowe 2005). Our findings provide a likely mechanism that explains previously reported viceroy unpalatability. These results are consistent with the Müllerian reclassification of this classic mimicry system.

Acknowledgments We thank J. Oliver for assistance in collecting the specimen; J. Gu, B. Jackson, R. Lindroth, C. Orians, and V. Rodriguez for assistance in chemistry; D. Bowers, D. Papaj, and D. Ritland for discussion; and the late Hon. M. Rothschild for enthusiasm and insight. This work was funded by an NSF Graduate Research Fellowship, an NSF Doctoral Dissertation Improvement Grant, a University of Arizona Center for Insect Science Graduate Research Grant, and a University of Arizona BIO5 Fellowship to K.L.P.

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J Chem Ecol (2007) 33:1160–1176
DOI 10.1007/s10886-007-9308-z

Immunological Memory of Mountain Birches: Effects of Phenolics on Performance of the Autumnal Moth Depend on Herbivory History of Trees

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Received: 26 February 2007 / Revised: 16 April 2007 / Accepted: 30 April 2007 /
Published online: 15 May 2007
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Abstract Plants have been suggested to have an immunological memory comparable to animals. The evidence for this, however, is scarce. In our study with the mountain birch—*Epirrita autumnata* system, we demonstrated that birches exposed as long as 5 yr to feeding of *E. autumnata* larvae (delayed induced resistance, DIR), responded more strongly to a new challenge than trees without an herbivory history. Pupal weights remained lower, and the duration of the larval period was prolonged in the DIR trees, although immunity, measured as an encapsulation rate, was not affected. We further demonstrated that the effects of birch phenolics on performance of *E. autumnata* were different in the exposed (DIR) trees from naive control trees, although we found only one significant change in chemistry. The quercetin:kaemferol ratio was increased in DIR trees, suggesting that herbivory caused oxidative stress in birches. In DIR trees, phenolics, especially hydrolyzable tannins (HTs), affected pupal weights negatively, whereas in control trees, the effects were either nonsignificant or positive. HTs also prolonged the duration of the larval period of females, whereas peroxidase (POD) activity prolonged that of males. We suggest that the causal explanation for the induced resistance was an enhanced oxidation of phenolic compounds from the DIR trees in the larval digestive tract. Phenolic oxidation produces semiquinones, quinones, free radicals, and ROS, which may have toxic, antinutritive, and/or repellent properties against herbivores.

Keywords Autumnal moth · Defense · *Epirrita Autumnata* · Herbivory · Hydrolyzable tannins · Immune defense · Induced resistance · Mountain birch · Peroxidases · Phenolic compounds

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Introduction

Herbivory-induced changes in plants have been intensively studied. These physiological and biochemical changes may increase (Danell et al. 1997; Karban and Baldwin 1997; Lempa et al. 2004) or decrease (Haukioja 1982; Karban and Myers 1989; Tallamy and Raupp 1991; Karban and Baldwin 1997) the palatability of plants and also their nutritive value for plant-eating animals. Induced resistance can be divided into rapidly induced resistance (RIR) that appears within the same season and delayed induced resistance (DIR) that affects herbivores in subsequent seasons (Haukioja 1982).

Herbivory triggers a sensitive signal cascade, which may lead to an increase in plant defense at the vicinity of wound sites and at distal, unwounded parts of plants (Ryan 2000). In plants, wounding may also trigger the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radicals (·OH), and singlet oxygen. H_2O_2 acts as a signal molecule, which activates defense-related genes like the ones encoding phenoloxidases (Levine et al. 1994; Orozco-Cárdenas et al. 2001; Gatehouse 2002). As ROS are highly reactive molecules capable of oxidizing membrane lipids, DNA, and proteins (Chan et al. 1999; Schweigert et al. 2001; Heim et al. 2002; Pourcel et al. 2006), they may also have direct defensive properties against plant-eating animals (Bi and Felton 1995).

The leaves of deciduous trees often contain a high diversity of phenolic compounds, some of which are defensive against plant-eating animals. Despite intensive research, the mechanisms behind defensive activity of phenolics have remained poorly understood. For example, the principal role of tannins traditionally has been thought to be the precipitation of proteins via hydrogen bonding. Although this might occur under certain conditions, such a mechanism is ineffective against some insect herbivores, many of which have alkaline midgut and/or surfactants in their gut fluid; these prevent the formation of insoluble protein–tannin complexes (Martin et al. 1987, Appel 1993). However, at high pH (e.g. in the midgut of caterpillars), some phenolics, such as ellagitannins, auto-oxidize into quinones that are highly reactive and also toxic compounds (Barbehenn et al. 2006). Quinones form covalent bonds with nucleophilic moieties of biomolecules, such as the functional groups of amino acids and proteins, making them indigestible (Felton et al. 1989). The oxidation of phenolics may also occur by enzymatic reactions catalyzed by plant polyphenol oxidases (PPOs) and peroxidases (PODs) (Felton et al. 1989; Felton and Duffey 1991; Bi and Felton 1995).

The population cycle of many northern Lepidopteran species is characterized by regular fluctuations, and populations may reach outbreak densities at certain intervals (Tenow 1972; Myers 1998). The driving forces behind these cycles are likely predation, parasitism, and the induced resistance of plants (Ruohomäki et al. 2000). The delayed induced resistance (DIR) that occurs after peak years might explain why population density remains low after the collapse of a population (Haukioja 1982; Haukioja et al. 1988). In contrast, induced amelioration may act as a positive regulator of population density in the increasing phase of the cycle (Ruohomäki et al. 2000). In a recent study with the mountain birch—autumnal moth system, RIR retarded growth of the autumnal moth (*Epirrita autumnata* Borkhausen) larvae, whereas DIR had no effect on growth parameters, but instead, increased the immunity measured as an encapsulation rate (Kapari et al. 2006), i.e., induced resistance may have enhanced the growth of a population by decreasing infection and parasitism.

In mountain birch (*Betula pubescens* ssp. *czerepanovii* (Orlova) Hämet-Ahti)), total phenolics have been shown to increase and total nitrogen to decrease due to herbivory

(Haukioja et al. 1985; Ruohomäki et al. 1996; Kaitaniemi et al. 1998; Ossipov et al. 2001). However, no single phenolic or group of compounds has been found unequivocally to be behind the induced resistance (Haukioja 2005). We have demonstrated that birch PPOs that oxidize phenols might contribute to the RIR of birches (Ruuhola et al., unpublished results).

Plants are suggested to have an immunological memory comparable to animals: repeated challenges by herbivores induce a more effective defense response than one-season challenges (Karban and Niiho 1995). Our purpose here was to test, whether a challenge conducted as long as 5 yr earlier still had an effect on the resistance response of mountain birches. This might explain why population density remains low after a population collapse. We utilized the same trees as Lempa et al. (2004) used 5 years earlier. We reared bagged larvae in both control and larval trees until they pupated and measured the immune defense and other life history traits of *E. autumnata*. To characterize the factors that are behind the resistance of trees against moth larvae, we determined phenolic contents and oxidase activities in birch leaves and correlated these with larval performance on control and DIR-trees.

Methods and Materials

Experimental Organisms The mountain birch inhabits subarctic and arctic areas in northern Fennoscandia, where it forms both the northern and upper tree lines. The economic value of mountain birches has not been acknowledged, whereas it has significant ecological value in subarctic systems (Wiegolaski 2005). The phenotype is highly variable from small persuading shrubs to large monocormic trees, probably due to the hybrid nature of the species (Vaarama and Valanne 1973). In our study area, the prevailing growth form is a low (ca 2 to 5 m) polycormic tree, whose canopy consists of multiple clonal ramets of both short and long shoots; the major part of the canopy is built from short shoots whose leaves flush simultaneously in early spring.

The autumnal moth, *E. autumnata*, is a univoltine, generalist capital feeder, whose larvae hatch synchronously with the bud burst of mountain birch (Kaitaniemi et al. 1997). Despite its generalist nature, the larvae feed mainly on leaves of mountain birches in northern latitudes, apparently due to the abundance (Seppänen 1970). Moth larvae get through five distinct instars before they pupate in the litter from the end of June to early July. Adult moths fly in autumn, and females lay either single eggs or small patches of eggs onto the lichen cover of trees. Autumnal moths are the worst pest species of mountain birches, and outbreaks occur periodically with 10- to 11-yr intervals in the Scandinavian mountain chain (Tenow 1972; Haukioja et al. 1988).

Experimental Design The experiment was conducted at Kevo Subarctic Research Station in the summer of 2005. We used the same trees that were exploited in an induction experiment 5 yr earlier (Lempa et al. 2004). In the summer of 2000, 24 trees growing in a river valley close to Kevo Subarctic Research Station (69° 45′ N, 27° 01′ E) were haphazardly selected for experimental trees (Lempa et al. 2004). Twelve trees were randomly selected for larval treatment trees, and the other 12 were left as unmanipulated control trees. Five ramets and two branches from each ramet were selected from each experimental tree. The selected branches were covered with large nylon mesh bags (ten bags/tree). We introduced 20 larvae onto one bagged branch, i.e., altogether 200 larvae per a larval treatment tree. In the summer of 2005, we randomly selected three ramets from each control and larval treatment tree. One large branch per each ramet was bagged (three bags/tree), and 12 newly hatched

first instar *E. autumnata* larvae were introduced into the bags at the time of the bud burst (36 larvae/tree). For practical reasons, we collected all larvae from mesh bags at the end of the last instar stage and reared them with the leaves of the experimental trees in a laboratory for a short period until they pupated (the pupation day was recorded for each pupa). One week after pupation, each pupae (7-d-old) was weighed, sexed, and the encapsulation rate was determined as described below.

Leaf phenology was recorded twice early in the season by using a classification based on bud morphology and leaf elongation (Alanen, unpublished results). Leaf samples for chemical analyses were collected when the bagged larvae were at their third and fifth instar stages (12th of June and 21st of June, respectively). On the first sampling day, leaves were young and rapidly expanding, and on the latter day, they were fully expanded. Leaf samples were collected from the three ramets with introduced larvae, and the leaves of one tree were pooled. Leaves were put into plastic bags on an ice bath and carried into a laboratory in a closed ice box for freeze drying. Leaf samples for enzymatic assays were collected on the latter day (21st of June) throughout the canopy, and ca 10 g (fresh weight) leaf material were collected. Samples were frozen immediately in liquid nitrogen and stored in −80°C before analysis.

Encapsulation Rate Assay The melanotic encapsulation depends on the activation of phenoloxidases (POs), which convert *o*-diphenols into quinones that subsequently polymerize to melanin (Söderhäll and Cerenius 1998). The encapsulation kills an intruder either by suffocation or through the production of necrotizing compounds (Nappi et al. 1995). In our study, the strength of insect immune defense was determined by measuring the melanotic degree of an encapsule formed around a foreign object (Kapari et al. 2006; Klemola et al. 2007). In the autumnal moth, the encapsulation rate correlates with the resistance against entomopathogenic fungi, which are known to cause substantial mortality in *E. autumnata* (Rantala and Roff 2007). In other species, the ability to encapsulate abiotic material strongly relates to the ability to encapsulate parasites (Paskewitz and Riehle 1994; Gorman et al. 1996).

A sterile nylon filament (2 mm long, 0.20 mm diameter rubbed with sandpaper) was inserted into the pupal hemocoel (Kapari et al. 2006). The pupae were incubated at + 14°C for 1 hr, after which the reaction was stopped by freezing the pupae in −80°C. The implant was photographed from three angles with a digital video recorder under a light microscope. The pictures were analyzed with Image Pro program with methods presented in Rantala et al., 2002.

Chemical Analyses Dried and ground birch leaves (20 mg material per sample) were extracted three times (3×1 hr) with 500 µl acetone/water (7/3, *v*/*v*) on a planary shaker. After extraction, acetone was evaporated from the combined extract *in vacuo* with an Eppendorf concentrator. The freeze–dried aqueous phase of the extract was dissolved in 1 ml water. The supernatant of the centrifuged (10 min at 2,500×*g*) sample was filtered through a 0.45-µm polytetrafluoroethylene (PTFE) filter and kept frozen at −20°C until analyzed with high-performance liquid chromatography with photodiode-array detection (HPLC-DAD).

HPLC-DAD analysis of birch leaf extracts was performed at 280 and 349 nm with Merck-Hitachi's LaChrom HPLC system (Merck-Hitachi, Tokyo, Japan). Column and chromatographic conditions were as described earlier (Salminen et al. 1999), except that 0.1 M H_3PO_4 was replaced with 0.05 M H_3PO_4. Phenolics were identified by comparing their retention times and ultraviolet (UV) spectra to those earlier reported by Salminen et al.

(1999, 2001). Phenolic compounds were quantified by using pedunculagin, pentagalloylglucose, catechin, coumaric acid, chlorogenic acid, and quercetin as external standards.

Enzymatic Assays Frozen birch leaves were ground with a cooled mortar and pestle, and 1.5 g leaf powder were immersed in 20 ml of cold extraction buffer containing 1 mM phenylmethanesulphonylfluoride (PMSF) (Sigma-Aldrich), 1 mM dithiothreitol(DTT) (Sigma), and 1% TritonX-100 (Acros) in 0.2 M potassium buffer, pH 7.5. We used 4.7% (*w*/*v*) of polyvinylpolypyrrolidone (PVPP) (Sigma) and 2.5% (*w*/*v*) of an anion exchange resin (AG® 1-X8 resin, 200–400 mesh chloride form, BioRad) in the extraction buffer to remove endogenous phenolic compounds. Leaf powder was homogenized in an Ultra Turrax homogenizer for 2×30 sec in a cold room (+8°C). Homogenates were incubated by shaking on an ice bath for 1 hr. Samples were centrifuged at 17,000×*g* for 20 min at +4°C. The supernatant was filtered through a cheesecloth.

Total POD activity was measured with 60 mM guaiacol (90%, Sigma) as a substrate and 20 mM H_2O_2 as a co-substrate, and the rate of change at A_{470} was followed (Ruuhola and Yang 2006).The increase in absorbance was followed for 3.5 min (initial reaction velocity) with a spectrophotometer with an external water bath to control the reaction temperature.

Combined PPO/POD activity was measured with 80 mM catechol (Sigma) as a substrate. The increase in absorbance during 10 min was read at 414 nm on a temperature controlled 96-well plate reader (Multiscan Ascent, Thermo Electron, Shanghai, China). The reaction was started by an addition of 10 μl of the enzyme extract. Pure PPO activity was measured similarly as combined PPO/POD activity but in the presence of a commercial catalase (Sigma, 280 U ml^{-1}), which removes H_2O_2 and inhibits POD activity. All enzymatic measurements were performed in triplicate at +25°C. The amount of protein in the samples was measured in triplicate by using the BioRad protein assay method, which is based on the Bradford method (Bradford 1976). Absorbance was measured with the plate reader at 595 nm. All enzyme activities were expressed as units per mg protein (U mg^{-1} protein), one unit being the amount of enzyme required to increase absorbance by 0.001 min^{-1}.

Statistical Analysis The effects of a previous defoliation treatment (conducted 5 yr earlier) on the pupal weight and the encapsulation rate of *E. autumnata* were tested with the mixed model analysis (SPSS 11.5.1.). The normality of the distribution and the equality of variances were tested with the *Kolmogorov–Smirnov's* and *Levene's* tests, respectively (SPSS 11.5.1.). The treatment and sex were set as fixed factors and the tree and ramet as random factors in the analysis; the tree was also set as a subject. There was no significant sex * treatment interaction, so it was removed from the final model. The effects of the treatment and sex on the length of the larval period were analyzed with the *Kruskall–Wallis's nonparametric* test, as the data was not normally distributed (SPSS 11.5.1.).

The effects of the treatment and leaf age (third or fifth instar leaves) on the contents of phenolic compounds were tested with the mixed model analysis (SPSS 11.5.1.). The treatment and leaf age (third or fifth instar leaves) were set as fixed factors, and the tree was set as a subject. There was no significant interaction between the treatment and leaf age, and it was removed from the final model. The normality of the distribution and the equality of variances were tested with the *Kolmogorov–Smirnov's* and *Levene's* tests, respectively (SPSS 11.5.1.). The contents of phenolics that were not normally distributed were *ln*-transformed to meet the assumptions of parametric tests. The effect of treatment on the activity of foliar oxidases (PPO, PPO/POD, and POD) was tested with one-way analysis of variance (ANOVA) (SPSS 11.5.1.).

Correlations between larval and leaf traits were tested with the *Pearson's two-tailed correlation* test (SPSS 11.5.1). The main idea was to test and present the differences in correlation coefficients between the control and the DIR treatments, not to present individual correlations (thus, *Bonferron* correction was not used). These differences were statistically tested by two independent analyses. First, the correlation coefficients were analyzed with the univariate analysis of ANOVA with the model, wherein the treatment, sex, leaf age (third or fifth instar leaves), and treatment * sex and treatment * leaf age were set as fixed factors. The dependent variables were correlation coefficients (calculated by correlating the pupal weight, development time, and encapsulation rate with phenolic compounds). In the analysis of the correlation coefficients of pupal weight and development time, the two way interactions were nonsignificant and were removed from the final model, but kept in the analysis of the correlation coefficients of the encapsulation rate. Second, we tested the differences in each correlation coefficient between the control and defoliation treatments by using the *Tukey's* type test presented in Zar (1999) (in chapter: "19.8 Multiple comparisons among correlation efficiencies").

Results

Effects of Previous Defoliation on Larval and Tree Traits Final pupal weights of *E. autumnata* remained lower in DIR trees that were exposed 5 yr earlier to the defoliation of *E. autumnata* larvae than in control trees (F=7.67, P=0.011) (Fig. 1a). In females, difference in pupal weights between control and DIR-trees was 9.5%, and in males, 6%. Development time of larvae was also longer (χ^2=10.93, P=0.001) in DIR trees than in controls (Fig. 1b). In contrast, encapsulation rate of pupae did not differ between control and DIR trees (Fig. 1c; F=0.08, P=0.783). Female pupae were heavier than male pupae (F=22.81, P<0.001), and the larval period of females was longer than that of males (χ^2= 28.84, P<0.001), whereas there were no sex differences in the encapsulation rate (F=0.40, P=0.530).

The activities of PPO, combined PPO/POD, or total POD did not differ significantly between treatments (Fig. 2a). Combined PPO/POD activity was higher than pure PPO activity (measured in the presence of commercial catalase), which suggests that the enzyme extract contained endogenous H_2O_2 (which PODs need as a co-substrate). In DIR trees, PPO/POD activity (and guaiacol POD activity) showed a slight (but nonsignificant) increasing tendency, whereas pure PPO activity showed a decreasing tendency, suggesting an increased endogenous level of H_2O_2 in DIR trees.

Neither did the contents of any individual phenolic compound nor the total contents of galloylglucoses, ellagitannins, hydrolyzable tannins, flavonoid glycosides, or total HPLC-phenolics differ significantly between control and DIR trees (data not shown). However, the quercetin:kaemferol ratio increased in DIR trees, although the change in the levels of quercetin or kaemferol were not statistically significant.

The contents of phenolic compounds varied significantly between sampling dates; generally the contents were markedly higher in young leaves of the third instar than in mature leaves of the fifth instar (Fig. 3). The contents of di- and trigalloylglucose (Fig. 3a), casuarictin, trigalloyl-HHDP-glucose, and galloyl-*bis*-HHDP-glucose, gallic acid, catechin (Fig. 3b), myricetin, quercetin, and kaempferol glycosides (Fig. 3c) decreased significantly during leaf development. In addition, the total contents of galloylglucoses, flavonoid glycosides, and HPLC-phenolics decreased toward leaf maturation (Fig. 3d). In contrast, the contents of

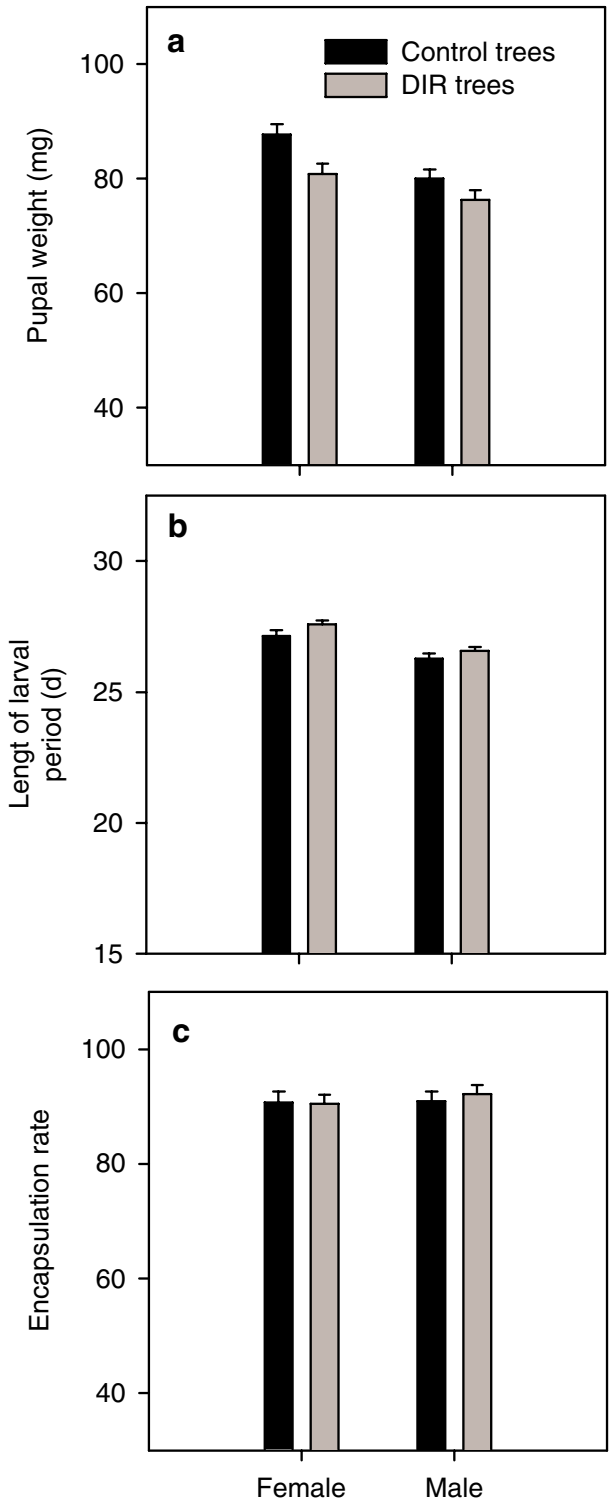


Fig. 1 Pupal weights (**a**), development time (**b**), and encapsulation rate (**c**) of *Epirrita autumnata* on control and DIR trees

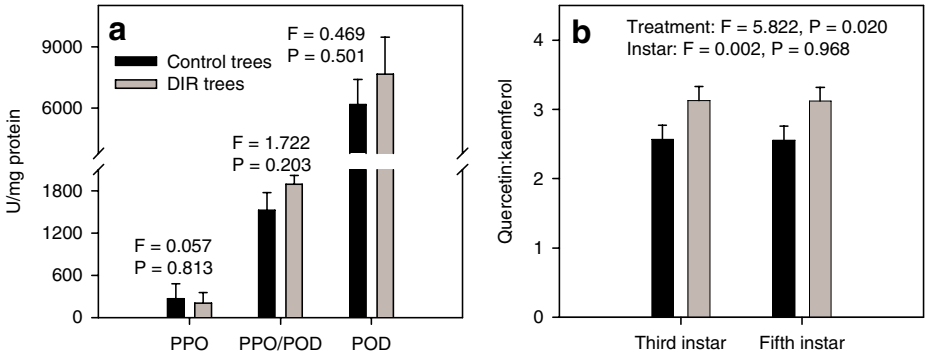


Fig. 2 Phenoloxidase activities (**a**) and the quercetin:kaemferol ratio (**b**) in the control and DIR trees. Pure PPO activity was measured by using catechol as a substrate, and POD activity was inhibited with commercial catalase. Combined PPO/POD activity was measured in the absence of catalase. Pure POD activity was measured with guaiacol and H_2O_2 as substrates

pedunculagin derivative, chlorogenic acids, and coumaroylquinic acids increased from young to mature leaves (Figs. 3a and c).

Relations of Tree Traits and Larval Traits between Treatments Although the contents of phenolic compounds did not differ between control and DIR trees, the correlation coefficients (calculated from correlations between larval parameters and the contents of phenolic compounds) differed significantly between the treatments (Table 1). The sexes did not differ in their correlation coefficients (Table 1). In the DIR treatment, correlations between pupal weight and contents of hydrolyzable tannins (HTs) were strongly negative, whereas in the control treatment these correlations were nonsignificant or positive (Table 2).

Correlations calculated between the length of larval period and contents of phenolics also differed; and sexes as well differed in their coefficients (Table 1). The length of the larval period of females correlated positively with the contents of tannic compounds in DIR trees, but the correlations were mostly nonsignificant or negative in control trees (Table 3). In other words, hydrolyzable tannins decreased the development rate of female larvae in DIR trees but had small effect in control trees and male larvae.

The correlation coefficients, calculated between the encapsulation rates of *E. autumnata* pupae and the contents of phenolic compounds in leaves, showed interesting features. The encapsulation rate of males was positively related to the contents of phenolics in the DIR treatment but negative in the control treatment (Table 4). The encapsulation rate of females, however, showed a positive relation to phenolic content in the control treatment, whereas correlations were negative in the defoliation treatment. Indeed, there was a significant treatment * sex interaction in the correlation coefficients (Table 1).

POD activities seemed to have differing effects on the performance of males (but not females) in DIR trees: the correlation between male pupal weight and POD activities was positive in the control (r=0.531, P=0.076) but nonsignificant in the larval treatment (r=−0.390, P=0.210). However, POD activity correlated positively with the length of male larval period in the DIR treatment (r=0.576, P=0.050), wherein the relation between male pupal weight and length of larval period was strongly negative (r=−0.845, P=0.001). In the control, both of these correlations were nonsignificant (data not shown). In addition, the correlations with other oxidase activities were nonsignificant.

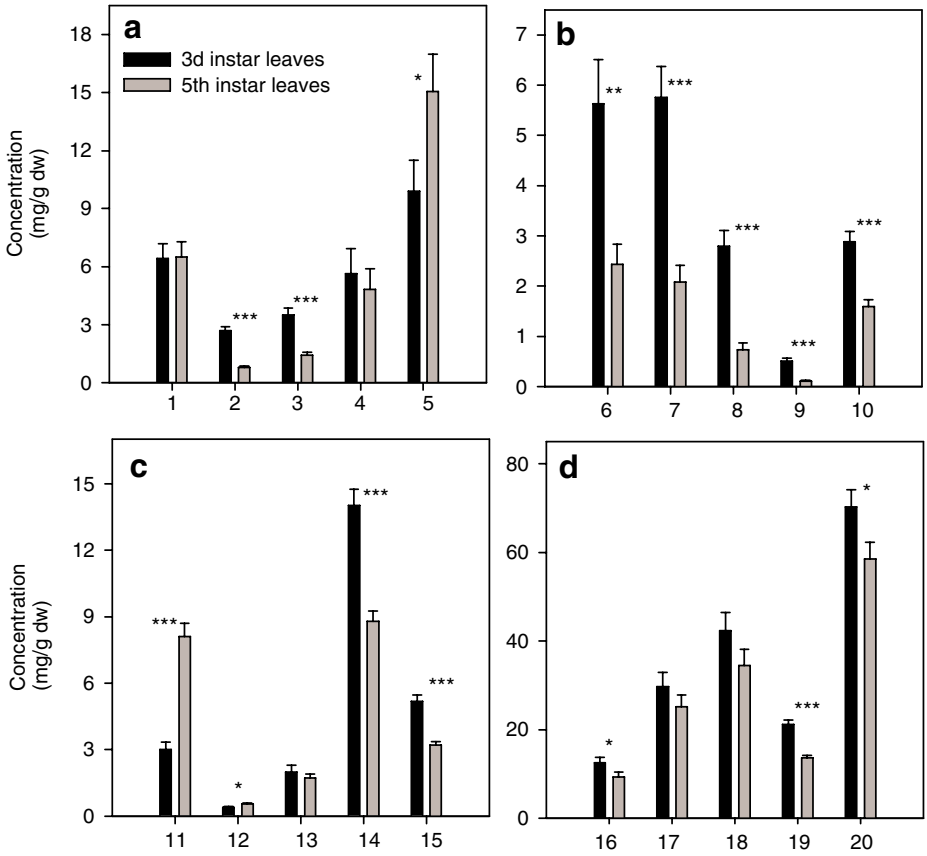


Fig. 3 Phenolic compounds in mountain birch leaves at the time of third instar (expanding leaves) and fifth instar (fully expanded leaves) of *E. autumnata*. **a** The contents of mono-, di- and trigalloylglucose (1-3), pedunculagin (4) and pedunculagin derivative (5). **b** The contents of casuarictin (6), trigalloyl-HHDP-glucose (7), galloyl-*bis*-HHDP-glucose (8), gallic acid (9), and catechin (10). **c** The contents of chlorogenic acids (11), coumaroylquinic acids (12), and myricetin, quercetin, and kaempferol glycosides (13-15). **d** The total contents of galloylglucoses (16), ellagitannins (17), hydrolyzable tannins (18), flavonoid glycosides (19), and HPLC-phenolics (20). The compounds whose contents differ significantly between these sampling points are marked with *asterisks* (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

POD activity correlated negatively with the encapsulation rate of males in control trees ($r=-0.611$, $P=0.035$) but not in DIR trees ($r=0.495$, $P=0.102$), whereas the combined PPO/POD activity correlated positively in control trees ($r=-0.598$, $P=0.040$). The encapsulation rate of males (but not females) was negatively associated to pupal weight both in the control ($r=-0.538$, $P=0.071$) and DIR trees ($r=-0.661$, $P=0.019$). The correlations between female fitness traits and phenoloxidase activities were nonsignificant in either treatment (data not shown).

Leaf phenology was negatively related to male pupal weight in control trees ($r=-0.592$, $P=0.043$), but not in DIR trees ($r=0.123$, $P=0.703$) and did not affect the traits of the females (data not shown). In addition, leaf water contents (determined both from the third

Table 1 Univariate analysis of the ANOVA on the differences in the correlation coefficients (performed between larval parameters and the contents of phenolics compounds) between treatments, sexes and the leaf age

Source		Pupal Weight	Development Time	Encapsulation Rate
Treatment	F	**18.407**	**32.536**	**7.429**
	P	**≤0.001**	**≤0.001**	**0.007**
Sex	F	0.931	**15.582**	0.218
	P	0.336	**≤0.001**	0.641
Leaf age[a]	F	0.982	1.860	0.255
	P	0.323	0.175	0.614
Treatment * sex	F	Ns	Ns	**48.125**
	P	Ns	Ns	**≤0.001**
Treatment * leaf age	F	Ns	Ns	**14.861**
	P	Ns	Ns	**≤0.001**

Statistically significant cases are in bold. Two-way interactions were nonsignificant in the case of correlation coefficients of pupal weight and development time and were removed from the model (marked ns).

[1] Leaf age=third or fifth instar leaf

and fifth instar leaves) showed no significant association to pupal weight or other traits (data not shown).

Discussion

This is the first study where the DIR has been shown to last as long as 5 yr, although it is a well-characterized phenomenon in the mountain birch (Haukioja and Neuvonen 1985; Ruohomäki et al. 1996; Kaitaniemi et al. 1998). In addition, our results suggest that the DIR is a specific process, not a passive response that results in the exhaustion of nutrients due to herbivory. Mild damages that occurred 5 yr earlier were not likely to have affected the reservoirs of trees in the present experiment. The definition of immunological memory of plants means that induced resistance should be stronger and more rapid in plants that are challenged a second time to an herbivore attack than in plants that encounter the challenge the first time (Karban and Niiho 1995; Baldwin and Schmelz 1996). Our results satisfy this definition as trees that were earlier exposed to herbivory reacted more strongly to a new challenge than trees without a marked herbivory history. We found that larval treatment, which was conducted as long as 5 yr earlier, still affected the performance of moth larvae: pupal weights remained significantly lower in DIR trees than in control trees, and also the length of larval period was prolonged on previously damaged foliage.

In a study that was conducted previously in the beginning of the increase phase of moth cycle, DIR did not affect growth rate or pupal mass of the autumnal moth; whereas the encapsulation rate of pupae was enhanced in DIR trees (Kapari et al. 2006). The present study, however, was conducted in the later stage of the cycle, and the results are opposite: DIR retarded pupal weight and prolonged the larval stage, whereas encapsulation rate was not affected. One explanation is the different timing of the second challenge: in the former study, the experiment was conducted 1 yr after the previous defoliation, whereas in the latter study, the experiment was conducted 5 yr after the first challenge. This may partly explain the discrepancies. Both birches and larvae may respond differently depending on the phase of the cycle. This hypothesis awaits confirmation.

Table 2 Correlation coefficients of the Pearson's correlation test performed between the pupal weight of *E. autumnata* and the contents of phenolics compounds in mountain birch leaves

Phenolic Compound	Third Instar Leaves				Fifth Instar Leaves			
	Females		Males		Females		Males	
	Control	DIR	Control	DIR	Control	DIR	Control	DIR
Total hydrolysable tannins	−0.248	**−0.702***	−0.028	**−0.555**	−0.207	−0.441	0.044	−0.312
Total galloylglucoses	−0.250	−0.174	−0.142	0.037	−0.340	−0.241	−0.027	−0.147
Monogalloylglucose	−0.338	0.128	0.087	0.355	−0.413	−0.042	−0.052	0.025
Digalloylglucose	−0.317	−0.426	0.082	−0.251	0.180	−0.323	0.034	−0.243
Trigalloylglucose	−0.137	**−0.602***	0.291	**−0.540**	−0.124	−0.452	*0.431*	*−0.506*
Total ellagitannins	−0.233	**−0.820*****	−0.080	**−0.716****	−0.149	−0.486	0.071	−0.353
Pedunculagin	0.088	**−0.634***	*0.336*	***−0.552***	−0.094	−0.439	0.305	−0.258
Pedunculagin derivative	−0.379	**−0.482**	−0.557	−0.427	−0.092	−0.233	0.001	−0.175
Casuarictin	*−0.117*	***−0.791****	*0.040*	***−0.731****	−0.138	−0.427	0.020	−0.375
Trigalloyl-HHDP-glucose	−0.340	**−0.530**	−0.047	−0.314	−0.154	−0.205	−0.093	−0.115
Galloyl-*bis*-HHDP-glucose	−0.258	**−0.565**	−0.135	−0.296	**−0.644***	**−0.738****	−0.111	**−0.716****
Total flavonoid glycosides	0.317	0.278	−0.282	0.078	**0.505**	0.284	−0.321	0.008
Myricetin glycosides	0.027	0.078	0.025	0.164	0.144	0.041	−0.071	−0.024
Quercetin glycosides	0.495	0.331	−0.082	0.127	**0.520**	0.274	−0.320	0.001
Kaempferol glycosides	0.089	−0.095	**−0.615***	−0.222	0.448	0.003	−0.325	−0.050
Precursors of tannins								
Gallic acid	−0.269	−0.096	−0.436	0.019	0.160	−0.279	0.011	−0.456
Catechin	0.025	−0.451	*0.370*	*−0.491*	−0.444	***−0.672****	−0.116	**−0.580***
Phenolic acids								
Chlorogenic acids	−0.253	0.147	−0.007	0.167	−0.005	0.171	−0.115	0.024
Coumaroylquinic acids	−0.337	−0.155	0.061	−0.273	−0.338	0.007	0.060	−0.282
Total HPLC-phenolics	−0.190	**−0.690***	−0.115	**−0.575***	−0.160	−0.357	−0.024	−0.299

Statically significant cases are marked with asterisks. Statistically close to significant cases ($P \leq 0.10$) are in bold. Coefficients that differ significantly between control and DIR-treatments are in italics. $n=12$

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

The nature of the mountain birch–autumnal moth interaction is complex (see Haukioja 2005): we did not find any individual inducible factor that alone could explain the retarded pupal weights on the induced trees. No individual phenolic compound was significantly affected by the treatment, nor did we find a significant increase in foliar enzyme activities. The only significant difference in the chemistry of control and DIR trees was the enhanced quercetin:kaemferol ratio in DIR trees. In addition, we found that correlations between phenolic compounds and larval traits differed significantly between the treatments. Correlations between the contents of phenolics (especially HTs) and pupal weights were negative in the DIR treatment and nonsignificant or positive in the control treatment, and the larval period of females was prolonged by phenolics, whereas those of males were

Table 3 Correlation coefficients of the Pearson's correlation test performed between the length of larval period of *E. autumnata* and the contents of phenolics compounds in mountain birch leaves

Phenolic compound	Third Instar Leaves				Fifth Instar Leaves			
	Females		Males		Females		Males	
	Control	DIR	Control	DIR	Control	DIR	Control	DIR
Total hydrolysable tannins	0.105	**0.666***	−0.398	0.255	0.216	**0.751****	−0.374	0.345
Total galloylglucoses	0.083	**0.715****	−0.317	−0.271	*0.210*	***0.885*****	−0.428	0.129
Monogalloylglucose	*0.110*	***0.603****	−0.215	**−0.537**	*0.191*	***0.817*****	**−0.535**	−0.007
Digalloylglucose	0.028	**0.638***	**−0.532**	0.069	−0.079	**0.689***	−0.158	0.301
Trigalloylglucose	0.143	**0.516**	−0.362	0.145	0.441	**0.755****	−0.168	0.478
Total ellagitannins	0.105	**0.567**	−0.400	0.425	0.212	**0.626***	−0.342	0.405
Pedunculagin	0.189	0.112	−0.031	0.255	0.255	0.080	0.051	0.161
Pedunculagin derivative	−0.033	0.370	−0.347	0.351	0.077	**0.544**	−0.464	0.327
Casuarictin	0.125	0.297	−0.326	0.389	0.273	0.272	−0.119	0.267
Trigalloyl-HHDP-glucose	0.066	**0.666***	−0.471	−0.154	0.088	**0.664***	−0.271	−0.025
Galloyl-*bis*-HHDP-glucose	*−0.101*	***0.803****	−0.471	0.001	0.342	0.160	0.005	**0.663***
Total flavonoid glycosides	−0.149	−0.186	0.472	0.108	−0.050	−0.133	**0.563**	0.083
Myricetin glycosides	0.229	−0.187	0.312	−0.273	0.240	0.023	0.447	−0.063
Quercetin glycosides	−0.106	−0.051	**0.509**	0.056	−0.069	0.027	0.483	0.084
Kaempferol glycosides	−0.398	−0.253	0.284	0.380	**−0.545**	−0.473	0.236	0.153
Precursors of tannins								
Gallic acid	−0.232	0.444	−0.324	0.015	−0.320	0.006	*−0.320*	***0.762****
Catechin	0.157	**0.528**	−0.335	0.257	0.300	**0.548**	0.005	0.492
Phenolic acids								
Chlorogenic acids	0.085	−0.154	0.116	−0.519	−0.382	−0.151	−0.277	−0.292
Coumaroylquinic acids	0.349	0.085	−0.020	0.130	0.301	−0.187	0.022	0.131
Total HPLC-phenolics	0.077	**0.676***	−0.262	0.263	0.146	**0.675***	−0.337	0.313

Statically significant cases are marked with asterisks. Statistically close to significant cases ($P \leq 0.10$) are in bold. Coefficients that differ significantly between control and DIR-treatments are in italics. $n=12$

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

prolonged by POD activity in DIR trees. Similar results where the negative correlations were found in larval treated trees but not in control trees have been reported in other studies with mountain birch (Lempa et al. 2004; Ruuhola et al. unpublished results) and its parental species *Betula nana* (Ruuhola et al. unpublished results).

Both the antioxidative and prooxidative capacity of flavonoids is proportional to the number of OH-groups in the B-ring (Heim et al. 2002), and quercetin has two, but kaemferol has only one. The ratio of dihydroxylated:monohydroxylated flavonoids has been shown to increase as a response to UV-B radiation in many plant species (Liu et al. 1995; Markham et al. 1998a, b; Ryan et al. 1998; Olsson et al. 1999), but this is possibly the first study wherein herbivory has been shown to affect the ratio. Artificial acid rain and heavy metal pollution also increase this ratio in the mountain birch (van Ooik et al., unpublished results).

The increase in the quercetin:kaemferol ratio in the leaves of DIR trees suggests an increased oxidative stress due to herbivory. Enzymatic data also indicate an increase in the endogenous level of H_2O_2. In an earlier study with mountain birch, we found that herbivory

Table 4 Correlation coefficients of the Pearson's correlation test performed between the encapsulation rate of *E. autumnata* and the contents of phenolics compounds in mountain birch leaves

Phenolic Compound	Third Instar Leaves				Fifth Instar Leaves			
	Females		Males		Females		Males	
	Control	DIR	Control	DIR	Control	DIR	Control	DIR
Total hydrolysable tannins	0.231	−0.145	−0.327	0.172	−0.003	−0.103	*−0.388*	***0.498***
Total galloylglucoses	0.414	−0.199	−0.258	−0.196	0.203	0.177	−0.248	0.276
Monogalloylglucose	**0.561**	−0.139	−0.088	−0.453	0.245	0.290	−0.255	0.001
Digalloylglucose	0.360	−0.176	−0.463	0.110	−0.155	0.064	−0.391	0.271
Trigalloylglucose	0.056	−0.343	−0.433	0.192	−0.380	0.342	***−0.581***	*0.344*
Total ellagitannins	0.160	−0.106	−0.329	0.292	−0.083	−0.211	*−0.432*	***0.546***
Pedunculagin	−0.178	−0.422	−0.228	0.249	−0.138	**−0.614***	−0.343	0.314
Pedunculagin derivative	0.270	−0.296	0.001	0.292	−0.123	−0.193	*−0.462*	***0.539***
Casuarictin	0.142	0.116	−0.113	0.146	−0.059	0.067	−0.052	0.162
Trigalloyl-HHDP-glucose	0.046	0.059	**−0.576***	−0.182	−0.010	0.303	−0.242	−0.033
Galloyl-*bis*-HHDP-glucose	0.313	0.008	−0.402	0.013	0.407	0.170	0.110	**0.698***
Total flavonoid glycosides	0.094	−0.362	0.470	0.312	−0.348	−0.234	0.148	**0.427**
Myricetin glycosides	−0.226	−0.115	0.272	−0.101	−0.232	−0.229	0.309	−0.061
Quercetin glycosides	−0.044	−0.281	0.367	0.230	−0.315	−0.219	0.044	0.376
Kaempferol glycosides	**0.509**	−0.286	**0.548**	0.409	−0.269	−0.007	−0.144	0.341
Precursors of tannins								
Gallic acid	0.041	0.381	−0.219	−0.165	0.080	0.089	***−0.622****	***0.763****
Catechin	−0.241	−0.238	***−0.597****	*0.451*	0.089	−0.293	0.175	**0.576***
Phenolic acids								
Chlorogenic acids	0.413	0.109	−0.125	−0.491	0.473	0.044	−0.406	−0.114
Coumaroylquinic acids	0.331	−0.083	0.020	0.224	0.387	−0.059	−0.230	0.370
Total HPLC-phenolics	0.297	−0.217	−0.237	0.226	0.051	−0.130	*−0.427*	***0.538***

Statically significant cases are marked with asterisks. Statistically close to significant cases ($P \leq 0.10$) are in bold. Coefficients that differ significantly between control and DIR-treatments are in italics. $n=12$

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$

caused oxidative stress in birch leaves: H_2O_2 accumulated in the vicinity of the wound sites, POD and PPO activities increased, and the activity of the antioxidative catalases that remove H_2O_2 decreased concomitantly (Ruuhola and Yang 2006). In other plant species, wounding also has been shown to increase the production of ROS (Levine et al. 1994; Bi and Felton 1995; Orozco-Cárdenas et al. 2001; Gatehouse 2002).

Despite the antioxidative and health-promoting effects of flavonoids and other phenolics, they may actually be prooxidative agents, e.g., in the presence of transition metals, NADH, ROS, oxygen, and oxidases (Hoover et al. 1998a, b; Chan et al. 1999; Schweigert et al. 2001; Heim et al. 2002; Pourcel et al. 2006). Oxidation of phenols, like flavonoids, by PPOs and PODs, produces quinones and semiquinones, which can, through coupled oxidation reactions, oxidize other polyphenols that cannot be directly oxidized by oxidases (Pourcel et al. 2006), i.e., quinones formed by oxidation of flavonoids may also activate the oxidation of HTs.

The toxicity of catecholic compounds containing two hydroxyl groups in the *ortho*-position is most often linked to the generation of ROS and damages in DNA and proteins (Schweigert et al. 2001). Differences in the oxidative status of leaf cells may explain the

observed phenomenon in birch: phenolics and phenoloxidases produce ROS and reactive radicals in larval trees, which are quenched in control trees. Which factors quench the oxidative stress in control trees is not yet known, but ascorbate is a good candidate, as a high level of ascorbate attenuates generation of ROS by phenolics and would regulate the prooxidant activity of phenolics *in vivo* (Heim et al. 2002).

Autumnal moth sexes did not differ in the strength of their immune defense, which is common in insects (Rantala et al. 2007 and references therein), although the components that affected the encapsulation rate differed between sexes. We know that in males, the genetic variation of immune response is largely additive or nondirectional with respect to dominance, whereas in females, variation is reduced and consists of a directional dominance variance (Rantala and Roff 2007). In addition, sexes differed in their correlations between life history traits: in females, the strength of immunity correlated positively with body size, whereas males' immune response increased with a reduction in development time (Rantala and Roff 2007). Thus, the genetic differences between males and females may cause differences in the effects of phenolic compounds on the immunity of males and females.

Interestingly, the chemicals (HTs, catechin, kaempferol glycosides) that had a negative effect on male pupal weight in larval trees affected encapsulation rate positively. There may be a trade off between body size and strength of immunity (Rantala and Roff 2005). Hoover et al. (1998a, b) found that bacoluviral infections of noctuid larvae *Heliotis virescens* and *Helicoverpa zea* were decreased by the phenolics' redox cycling catalyzed by PODs, a process that also involved the generation of H_2O_2. They did not, however, study whether immunity of larvae was affected by phytochemicals and/or the activity of foliar oxidases.

Rapid developmental changes, together with high among and within tree variation in both secondary and primary compounds in mountain birch leaves, have complicated the search of induced defensive factors (Haukioja et al. 1988; Kause et al. 1999; Haukioja et al. 2002). Condensed tannins increase with leaf maturation, whereas flavonoids and hydrolyzable tannins decrease (Salminen et al. 2001, 2002; Riipi et al. 2002). In this study, we found that the contents of several individual HTs, flavonoids, and total HPLC-phenolics similarly decreased during leaf maturation, whereas chlorogenic acid, coumaroylquinic acids, and pedunculagin derivative increased.

Haukioja et al. (2002) found that specific defensive leaf traits are sensitive to simultaneous variation in other leaf components and that different instars might be vulnerable and affected by different factors. Here, the contents of HTs determined from young third instar leaves negatively correlated with final pupal weight in the DIR trees, suggesting that early phases in larval development are critical for final pupal weights, although most of the weight gain is attained during the last fifth instar (see also Tammaru 1998). In third instar leaves, the contents of HTs were significantly higher than in the fifth, further indicating a defensive role of HTs against early instars (see also Kaitaniemi et al. 1998). The leaf maturing index is an important determinant of tree suitability for autumnal moth larvae (Haukioja et al. 2002), and fast larval development is possible only in young leaves (Kause et al. 1999). In our study, the phenological index of trees was negatively related to male pupal weight in control trees but not in DIR trees, indicating that other leaf traits were more important determinants for the growth of larvae on induced foliage than the leaf maturation index.

In summary, our results show that mountain birches possess a long-term memory: trees that are exposed to herbivory 5 yr earlier are more strongly defended than control trees without an herbivory history. How the immunological memory functions in the molecular level, needs to be investigated. The causal mechanism behind the induced resistance is

suggested to be an enhanced oxidation of phenolic compounds in larval trees due to increased oxidative stress. In our proposed model, herbivory enhances the accumulation of ROS, activates foliar phenoloxidases, and exhausts the antioxidants. These oxidation products, quinones, semiquinones, and ROS may have toxic, antinutritive, and repellent properties against herbivores.

Acknowledgements The research was conducted in Kevo Subarctic Research Station, and we thank the staff for help and co-work. We also thank the department of Physiology and Genetics for providing the facilities for the enzymatic assays. We also thank our field assistants Ulla Anttila, Tia Lampela, Liisa Rantala and Mikko Oikamo, and Piia Saarinen for doing the phenolic analyses. Salla-Riikka Vesterlund kindly checked the English language. The study was supported financially by the Academy of Finland, Kone and Emil Aaltonen Foundation.

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J Chem Ecol (2007) 33:1177–1185
DOI 10.1007/s10886-007-9283-4

Predatory Behavior of *Polistes dominulus* Wasps in Response to Cardenolides and Glucosinolates in *Pieris napi* Caterpillars

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Received: 11 July 2006 / Revised: 20 February 2007 / Accepted: 3 March 2007 /
Published online: 24 April 2007
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Abstract To examine how plant allelochemicals in prey affect foraging choices made by generalist predator paper wasps, *Polistes dominulus* (Vespidae), we compared predation on *Pieris napi* (Pieridae) caterpillars reared on host plants with different allelochemicals. In naturalistic behavioral choice experiments, free-flying wasps chose between living pierids reared on cabbage (*Brassica oleracea*), which lacks deterrent allelochemicals, or alternate host plants with potentially deterrent allelochemicals. The alternative host plants were: wormwood mustard, (*Erysimum cheiranthoides*: Brassicaceae), which contains cardenolides; nasturtium (*Tropaeolum majus*: Tropaeolaceae) with high concentrations of chlorogenic acid; and black mustard (*Brassica nigra*: Brassicaceae) with high concentrations of the aliphatic glucosinolate, sinigrin. Although wasps captured equal numbers of caterpillars reared on cabbage and each alternate host plant, they spent significantly longer handling prey from the alternate host plants as they selectively removed the caterpillar's gut, which contained the plant material. This was true even if the caterpillar did not sequester toxins in its tissues, as revealed by high performance liquid chromatography (HPLC) analysis of *Erysimum*-reared pierids. Because handling time is longer, predators that capture pierids containing non-sequestered allelochemicals experience an overall reduction in foraging rate that may translate into a fitness cost.

Keywords Predatory behavior · Cardenolides · Glucosinolates · *Polistes dominulus* paper wasps · Pierid larvae · Tritrophic interactions · Handling time · Sequestration

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Introduction

Plants have evolved a wide variety of chemical defenses against herbivory. Although plant allelochemicals may effectively deter herbivory by most insect species, in many cases, insect specialists are able to consume the plants, and subsequently use the acquired allelochemicals as defense against predators (Price et al. 1980; Brower 1984). The literature on the effectiveness of allelochemicals acquired from host plants in reducing predation on insect prey has focused on vertebrate predators of adult insect herbivores (see Brower 1984), or effects of allelochemicals on parasitoids (Duffey et al. 1986; Barbosa 1988; Gauld and Gaston 1994). The effects of acquired allelochemicals in prey on generalist invertebrate predators have also received attention, although generalist predators are major selective forces on immature insect herbivores (Brower 1984; Stamp and Bowers 1988; Dyer 1995; Müller and Brakefield 2003; Boeve and Müller 2005). Generalist predators are proposed to play a significant role in restricting host-plant range of insect herbivores by selectively predating more on polyphagous generalists than on specialists (Bernays 1988; Bernays and Graham 1988). Relatively little is known, however, about the dynamics of how generalist invertebrate predators deal with potentially toxic allelochemicals in insect herbivores.

We developed a predator–herbivore–plant system to examine how generalist paper wasp predators respond to herbivorous caterpillars raised on host plants with different types of defensive chemistry (Eigenbrode, et al. 2000; Rayor and Munson 2002; Rayor 2004). The relatively large, social paper wasps, (Vespidae: *Polistes* spp.), are major generalist predators on lepidopteran larvae that they capture to feed to their carnivorous larvae (Raveret Richter 2000; Rayor 2004). Because the adults feed solely on nectar, their foraging behavior is dictated by the nutritional requirements of their developing larvae (Raveret Richter 2000). We have previously demonstrated that *Polistes dominulus* prefer prey with lower concentrations of allelochemicals (Danaidae: *Danaus plexippus*: Rayor and Munson 2002; Rayor 2004) and that larval development is slower when reared on caterpillars raised on host plants with high cardenolide concentrations (Rayor, unpublished data). While palatability may be a key factor affecting prey capture, the wasps also must locate prey on host plants that may differ considerably in leaf structure or density. Plant architecture has been shown to affect the ability of wasps to locate or capture caterpillars (Cornelius 1993; Geitzenauer and Bernays 1996. Eigenbrode et al. 2000).

In this study, we used a behavioral choice test to determine whether the predatory behavior of *P. dominulus* is affected by allelochemicals found in pierid caterpillars' host plants. We hypothesized that wasps would discriminate against conspecific *Pieris napi* (Pieridae) caterpillars that had been reared on host plants that differed in their defensive compounds. The oligophagous feeding habits of *P. napi* permit a comparison of wasp predation on caterpillars reared on host plants with and without cardenolides or on host plants with different glucosinolate profiles, such as cabbage, nasturtium, and black mustard. The use of a single caterpillar species in these tests minimized behavioral or morphological factors that could affect predation risk by wasps. Most pierids readily consume cabbage (*Brassica oleracea*) that contains indole glucosinolates, but *P. napi* also eats the crucifer, *Erysimum cheiranthoides*, which contains cardenolides that deter other *Pieris* species (Sachdev-Gupta et al. 1993; Renwick 2002). Nasturtium (*Tropaeolum majus*: Tropaeolaceae) contains a benzylic glucosinolate attractive to many cruciferous herbivores, but also contains high concentrations of chlorogenic acid unpalatable to some pierid species (Renwick and Huang 1995). Black mustard (*Brassica nigra*) contains high concentrations of the aliphatic glucosinolate, sinigrin (Fenwick et al. 1983).

Our goals were: (1) to examine how plant allelochemicals in prey affect foraging choices made by generalist paper wasps; and (2) to determine if the wasps use behavioral strategies to effectively overcome protection that caterpillars derive from the host plants allelochemicals.

Methods and Materials

Subjects Queens of the invasive European paper wasp, *P. dominulus* (Vespidae) with their six to ten cell nests were collected in early June 1995 and 1996 in Ithaca, New York. Nests were hung in a 5.8 by 2.7 m room in the Entomology Insectary Greenhouse on the Cornell University campus. As nests were captured at the single foundress stage with eggs and a few larvae, all workers that emerged from each nest were raised entirely in the experimental environment of the greenhouse and had foraging experience only with prey species provided to them. The first cohort of wasp larvae was fed highly palatable cabbage loopers (Noctuidae: *Trichoplusia ni*) raised on artificial wheat germ diet. Experiments started upon the emergence of the first cohort of adult foragers on 4 July 1995 and 3 July 1996. Each adult wasp was individually marked with enamel paint. Each summer, the *P. dominulus* wasps came from four nests. The number of developing larvae increased over the summer, from approximately 6 to 30 larvae per nest with 11 to 20 active foragers at a time. The exact number of foragers is provided in the results for each experiment. Experiments I, III, and IV were done in 1995, Experiment II in 1996. Individual foragers were active for several weeks before retiring, and a few were active foragers during two experiments in 1995.

Caterpillars and their host plants were raised in the greenhouse. Depending on the particular experiment, *P. napi* oleracea larvae were raised on cabbage (*B. oleracea capitata*, wormseed mustard (*E. cheiranthoides*), black mustard (*B. nigra*), or nasturtium (*T. majus*). Conspecific caterpillars raised on different host plants are referred to as different "types".

Behavioral Experiments Predatory bouts in *Polistes* involve prey detection, attack (initial contact), capture (subduing and killing), handling (cutting it into two or more pieces small enough to be carried in flight), departure (flying to the nest with prey pieces to be fed to the developing wasp larvae), and rapidly returning (to the site where "remains" of the prey have been left behind or where hunting has been successful) (Rabb and Lawson 1957; Raveret Richter 2000). Wasps accept or reject the prey during the attack, capture, and return phases. This study focused on the number of predation events ("captures"), duration from the initial attack until an individual wasp departed ("handling time"), and her return for prey remains ("remains"). "Total prey captured" reflects the number of prey pieces returned to the nest (all prey pieces taken from the initial capture and remains, minus the number of prey that were rejected during handling; see Rayor and Munson, 2002 for details). All prey used were sufficiently large that they needed to be cut into smaller pieces to be flown to the nest. Detailed behavioral data were recorded for 1-h sessions using the Noldus Observer 3.0 software on Psion hand-held computers. Focal observations were simultaneously recorded for five to ten individual wasps.

To compare wasp predatory behavior and prey preferences, free-flying wasps were given a choice between two types of caterpillars reared on different host plants for 1-h sessions. Approximately six size-matched live caterpillars of each type were widely distributed on six to eight presentation plants placed within 1 m of each other. Caterpillar numbers were held constant, by adding new caterpillars after others were killed but by placing them on different presentation plants and changing the location of presentation plants every half-hour. Care was taken not to attract the attention of foraging wasps when caterpillars were replaced. To control

for differences in plant architecture, the caterpillar's host plant ("own host") and cabbage were used as presentation plants in experiments I and II, but only cabbage in experiments III and IV. Presentation plants were replaced between sessions to minimize visual or olfactory cues from caterpillar hemolymph or leaf damage. Herbivory on presentation plants was minimal.

Behavioral choice experiments:

Experiment I: *P. napi* raised on *Erysimum* vs *P. napi* raised on cabbage, presented to wasps on their "own host" plants or on cabbage;

Experiment II: *P. napi* raised on cabbage and painted on the epidermis with 0.5 mg digitoxin/ml in methanol vs *P. napi* painted on the epidermis with methanol alone, presented to wasps on cabbage. Digitoxin is a representative cardenolide;

Experiment III: *P. napi* raised on nasturtium vs *P. napi* raised on cabbage, presented to wasps on their "own host" plants or on cabbage;

Experiment VI: *P. napi* raised on black mustard vs *P. napi* raised on cabbage, presented to wasps on cabbage.

Data Analysis As in nature, individual foragers made repeated prey captures. To avoid pseudoreplication issues, nonparametric Wilcoxon-signed rank tests were used to determine the direction of preferences by individual wasps for one prey type over the other in total prey returned to the nest (Seigel and Castellan 1988). The Wilcoxon-signed rank test evaluates the relative magnitude and direction of difference within paired samples for an individual wasp. Wilcoxon Signed Rank T values were reported for small sample sizes ($N<15$), Z values for large sample sizes ($N>15$).

Mean handling time for each prey type for individual wasps was compared with the nonparametric Wilcoxon Signed Rank Test (Seigel and Castellan 1988). Variation in intra- and interindividual handling time was large. We attribute the large variation in processing to differences in individual experience, motivation relative to the number of feeding larvae at the nest, or event-specific circumstances. Such circumstances include an unstable location on the plant where the prey is killed, necessitating dragging of the prey to a more stable location or the influence of other foraging wasps in the vicinity (which increases the speed of handling). Only predatory bouts greater than 30 s (from initial attack of live prey to departure for the nest with the prey ball) were used. Predatory bouts where wasps were interrupted by another wasp were omitted from handling time comparisons and inexperienced wasps that attacked fewer than four caterpillars were also omitted. Handling data were pooled for presentation plants in experiments I and II.

Overall differences in handling time for caterpillars raised on *Erysimum*, nasturtium, and black mustard compared to those raised on cabbage were calculated as follows: For each prey type, the mean difference between handling times was calculated for each individual wasp and transformed into a percentage. A grand mean of the individual percentages was subsequently calculated and is plotted in Fig. 2 along with 1 SD. The handling times for cabbage caterpillars were calculated only from those captured in each separate experiment and were not combined between experiments.

Chemical Analysis To determine whether *P. napi* sequestered cardenolides from *Erysimum*, HPLC was used and the cardenolide content examined from 1.97 g fresh *Erysimum* leaves, 0.184 g frass from *P. napi* raised on *Erysimum*, 0.33 g starved *P. napi* larvae raised on *Erysimum*, and 0.38 g *P. napi* pupae raised on *Erysimum* using the methodology of Sachdev-Gupta et al. (1993).

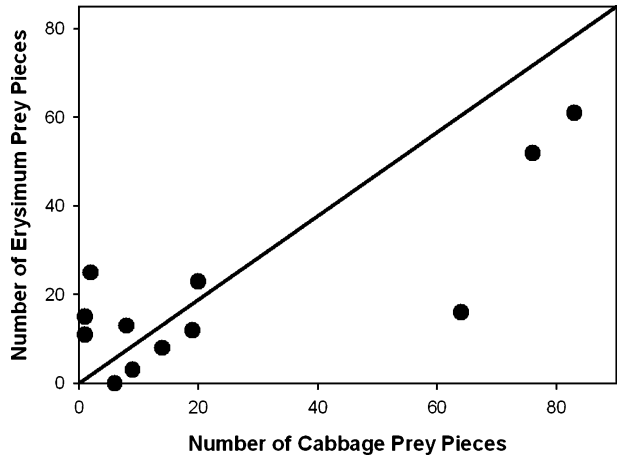


Fig. 1 Types of prey returned to nests by wasps. Individual foraging wasps took similar numbers of prey pieces of *Erysimum*-reared and cabbage-reared *Pieris napi* back to their nest. The *diagonal line* indicates equal numbers of each prey type. *Data above the line* indicate that an individual wasp captured more *Erysimum*-reared prey; *data below the line* indicate that the wasp captured more cabbage-reared prey. Data were pooled from both sets of presentation plants

Results

Behavioral choice: *P. napi* raised on *Erysimum* vs *P. napi* raised on cabbage

Equal numbers of *P. napi* raised on cabbage or on *Erysimum* were captured by individual wasps when presented on their own host plants [total prey captured: *Erysimum*-reared (N=84) and cabbage-reared (N=107); Fig. 1, T=10, foraging wasps N=5, P=0.31]. Similarly, when both types of caterpillars were simultaneously presented on cabbage presentation plants, there was no significant difference in number of captures [total prey captured: *Erysimum*-reared (N=159) and cabbage-reared (N=180); Fig. 1, T=42, foraging wasps N=11, P=0.23]. Only three wasps were active foragers during experiments on both sets of presentation plants.

Wasps meticulously removed the guts of *Erysimum*-reared caterpillars when processing prey; they did not remove guts of caterpillars raised on cabbage. This activity increased individual forager's handling time of *Erysimum*-reared caterpillars over cabbage-reared caterpillars (T=38.5, foraging wasps N=9, P=0.04, pooled presentation plant data). The

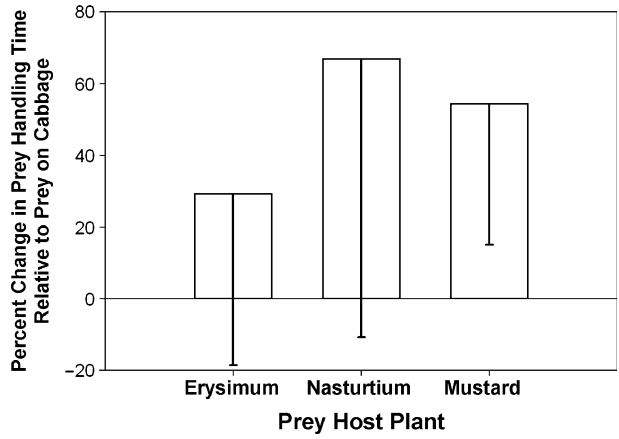


Fig. 2 Handling time by wasps as a function of prey host plant. Wasps spent more time handling *Pieris napi* prey raised on *Erysimum*, nasturtium, or black mustard host plants than on cabbage-reared prey, as indicated by the overall mean percent difference in time spent on each type of prey. *Each bar* represents separate experiments where the cabbage-reared prey was paired with an alternate host plant-reared prey. *Error bars* indicate standard deviation

overall mean handling time for all wasps was 29.3% longer on *Erysimum*-reared caterpillars than on cabbage-reared caterpillars (Fig. 2).

Chemical Analysis HPLC revealed peaks that were characteristic of cardenolides (Dimock et al. 1991) in the *Erysimum* leaf control sample and in the sample of frass from *Erysimum*-raised *P. napi*. Retention times and the total ultraviolet (UV) spectra were consistent with *Erysimum* cardenolide samples run under the same conditions (Sachdev-Gupta et al. 1993). Although starved before analysis, samples of larvae gave small peaks with absorption maxima at 221 and 222 nm, indicating the presence of traces of cardenolides. Pupae did not contain detectable cardenolides. Because the gut content of caterpillars is completely voided before pupation, the lack of cardenolides after pupation demonstrates that no sequestration occurred.

Behavioral Choice: *P. napi* with Digitoxin vs untreated *P. napi*

Prey pieces from pierids with the cardenolide standard digitoxin on their epidermis were returned to the nest far less (N=65) than pierids without the cardenolide (N=116). Wasps captured fewer caterpillars with digitoxin (Fig. 3, Z=2.66, foraging wasps N=18, P=0.004). Pierids with digitoxin were 3.6 times more likely to be rejected once bitten, and prey remains without digitoxin were handled 1.9 times more often. The time wasps spent handling pierids with digitoxin did not differ significantly from the time spent handling pierids without digitoxin (T=25, foraging wasps N=8, P=0.19).

Behavioral choice: *P. napi* raised on nasturtium vs *P. napi* raised on cabbage

Nasturtium contains the aromatic benzylic glucosinolate (glucotropaeolin) and high levels of chlorogenic acid. There was no significant difference, however, in the number of prey pieces taken by individual wasps from nasturtium- or cabbage-reared *P. napi* when caterpillars were presented on their own host plant [total prey captured: nasturtium-reared (N=56) and cabbage-reared (N=71); Z=1.17, foraging wasps N=16, P=0.12] or when they were presented on cabbage [total prey captured: nasturtium-reared (N=52) and cabbage-

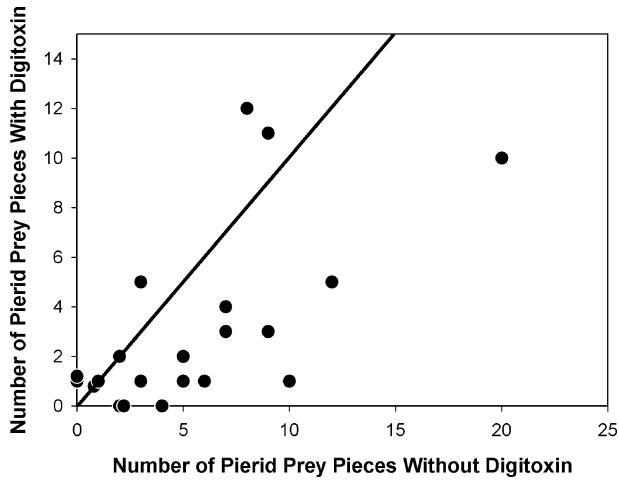


Fig. 3 Prey species taken by wasps with or without digitoxin. Individual wasps took significantly fewer *P. napi* caterpillars painted with digitoxin than those without digitoxin, as indicated by the data points below the diagonal line

reared (N=63); Z=0.84, foraging wasps N=20, P=0.20]. However, the time individual foragers spent handling caterpillars raised on nasturtium was greater than for caterpillars raised on cabbage (Z=2.72, N=17, P=0.02, pooled data). On average, wasp handling time on nasturtium-reared caterpillars was 66.8% longer than for those raised on cabbage due to time spent removing caterpillar guts (Fig. 2).

Behavioral choice: *P. napi* raised on black mustard vs *P. napi* raised on cabbage

Wasps found pierids raised on black mustard, which contains high concentrations of sinigrin, to be equally palatable to pierids raised on cabbage. There was no significant difference in the number of captures by individual wasps when these caterpillars were presented on cabbage (total prey captured: mustard-reared N=57, cabbage-reared N=50; Z=−0.933, foraging wasps N=16, P=0.17). Although relatively few wasps handled both caterpillar types more than once without interruption, handling time was longer on black mustard (T=21, foraging wasps N=6, P<0.015). Overall, wasps took 54.3% longer processing mustard-reared caterpillars than cabbage-reared caterpillars due to gut removal (Fig. 2).

Discussion

The results of the study indicate that *Polistes* wasps are not deterred from capturing *P. napi* that have consumed host plants containing cardenolides and glucosinolates in *Erysimum*, glucotropaeolin and chlorogenic acid in nasturtium, or sinigrin in black mustard. However, sensitivity to these allelochemicals is indicated by the wasps' significantly longer handling time of *P. napi* that had fed on these plants. The behavioral strategy of removing guts containing plant material from caterpillars that have not sequestered the allelochemicals effectively removed toxins that might affect larval development. Costs are likely incurred by this behavioral strategy. In the field, fewer prey items would be brought back to the nest because it takes more time to handle prey containing potentially toxic allelochemicals than prey without the compounds. Similarly, a wasp that spends longer time with prey at the host plant is more likely to attract other foragers' attention and risks fighting over the prey and possibly having wasps from other nests steal it (Raveret Richter 1990, personal observation). This could lead to a slower growth rate for developing wasp larvae, and fewer workers and reproductives produced over the course of a summer. Locating prey in the field is difficult and time-consuming, and rapid prey handling helps to maximize foraging success over the course of a day. Thus, a cost in slower prey handling due to plant allelochemicals may translate into an overall cost in fitness for a wasp colony.

P. dominulus were strongly deterred in this study by digitoxin that was painted on the epidermis of pierids. Most insect herbivores are strongly deterred from consuming *Erysimum* due to its high cardenolide content (Dimock et al. 1991; Sachdev-Gupta et al. 1993). The cardenolide concentrations are as high as those documented for milkweed (*Asclepias*) species (Rodman et al. 1982). We predict that if *P. napi* were able to sequester cardenolides from the *Erysimum* host plant in their tissues, the allelochemicals could function as a more effective antipredator defense. Based on our HPLC analysis, traces of cardenolides may be retained by *P. napi* larvae, but the allelochemicals are not stored or accumulated, so that none is detected in the pupae. *P. rapae* and *Pieris brassicae* have been shown not to sequester sinigrin (aliphatic glucosinolate) or several other benzylic or indole-glucosinolates (Müller et al. 2003). Pierids are not unique among lepidopteran larvae that feed on host plants containing potent allelochemicals in their lack of sequestration of the

chemicals (Bowers and Stamp 1997; Müller and Brakefield 2003). In contrast, lepidopteran larvae that do sequester a variety of allelochemicals from their host plants successfully reduce predation risk from generalist predators, including predatory wasps (Bernays 1988; Bernays and Cornelius 1989; Montllor et al. 1991; Stamp 1992; Dyer and Bowers 1996; Müller and Brakefield 2003). *P. dominulus* discriminates against high cardenolide levels in monarch caterpillars (Rayor 2004). Because the monarchs accumulate cardenolides in their hemolymph and epidermis (Duffey 1980), wasps are unable to avoid cardenolides by removing the guts alone. Monarch caterpillars were never gutted nor were their epidermis or other tissues selectively removed during processing (Rayor, unpublished data).

This study demonstrates that predatory wasps may use behavioral strategies to overcome potentially toxic host plant allelochemicals in prey.

Acknowledgments The work was supported by NSF DEB - 97 27547 to LSR and by NSF DEB- 9419797 to JAR. We thank Laurel Southard and the Cornell Howard Hughes Scholars Program for two summers of financial support for LJM. K. Campbell helped with 1996 data collection. Kimberly Lopez's assistance to LJM with chemical analyses was indispensable. The project and manuscript were improved by suggestions from Nancy Stamp, Lincoln Brower, Paul Feeny, Caroline Müller, Connie Rayor, and an anonymous reviewer. Particular thanks go to Elizabeth Bernays, University of Arizona, for initially encouraging LSR's approach to wasp predatory behavior. Patrick Hughes provided cabbage loopers. Brian Traw helped collect black mustards. Andrew Scherrer, Biostatistical Consultant, Biometrics Unit, Cornell University contributed statistical advice.

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J Chem Ecol (2007) 33:1186–1196
DOI 10.1007/s10886-007-9292-3

Feeding Selectivity by Mantled Howler Monkeys (*Alouatta palliata*) in Relation to Leaf Secondary Chemistry in *Hymenaea courbaril*

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Received: 22 November 2006 / Revised: 9 March 2007 / Accepted: 20 March 2007 /
Published online: 14 April 2007
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Abstract This study is a quantitative examination of primate feeding selectivity in relation to secondary chemistry within a single plant species, *Hymenaea courbaril*. It provides the first evidence that sesquiterpenes may act as feeding deterrents in mantled howler monkeys. A free-ranging group of mantled howler monkeys at the study site of Sector Santa Rosa, Area de Conservacion Guanacaste, Costa Rica were observed for the 2-month period of *H. courbaril* leaf flush in 1999. Tree characteristic data and leaf specimens were collected from 22 focal trees. Gas chromatography and mass spectrometry were used to estimate relative percentages of sesquiterpenes in leaf specimens. The monkeys fed only on the youngest leaves and only from particular trees. Whereas leaf stage selectivity was likely governed by tannin content and structural carbohydrates in younger and older leaf stages, respectively, differential tree use may be related to variability in sesquiterpene content. There is evidence that α-copaene may have played a role in interindividual tree use, and that cyperene may also be implicated. However, there is no reported evidence of antiherbivore activity for cyperene.

Keywords Primates · Howler monkeys · Feeding selectivity · Secondary chemistry · Sesquiterpenes

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Introduction

Much research has been conducted in the area of primate feeding selectivity in relation to plant primary and secondary chemistry (see Milton 1998 for an overview). However, in relation to secondary chemistry, research has not advanced much beyond the role of phenolics and alkaloids, and analyses have often been qualitative rather than quantitative (see Calvert 1985 and Berry 1998 for exceptions). The limited focus on these two groups of compounds is puzzling because (1) plants produce a vast array of secondary compounds; (2) many families of secondary compounds have suspected or demonstrated antiherbivore functions; and (3) herbivorous primates feed from a variety of different plant families, many of which differ greatly in their chemistry.

This research is an examination of feeding selectivity in relation to secondary chemistry within a single plant species, *Hymenaea courbaril*, by mantled howler monkeys. The study was not confounded by the many differences between plant species or by other factors involved in primate dietary selection, e.g., food availability and preferences; plant density, location, phenology, production, and abundance; learned local traditions, etc. It is the first time that sesquiterpenes have been considered to play a role in primate dietary choice.

Mantled howler monkeys are generalist herbivores that exhibit pronounced inter- and intraspecific dietary selectivity for plant parts and stages (Milton 1998). They are thought to choose plant parts in a way that maximizes/balances nutritional intake, whereas minimizing intake of secondary compounds (Glander 1978, 1981; Milton 1978; Estrada 1984). In terms of leaf choice, Milton (1979) reports that the protein-to-fiber ratio is the most important determinant. Glander (1981, 1982) has provided evidence that some differential species/tree/leaf stage selectivity is related to the secondary chemistry of leaves (especially the presence/absence of condensed tannins and alkaloids). Milton (1998) suggests that whereas there is little evidence that secondary chemistry plays a role in primate dietary selectivity, detoxification is energetically demanding and animals might be expected to avoid foods that are relatively costly.

Mantled howler monkeys feed on leaves from some trees and not others within particular food species (Milton 1980; Glander 1981; Welker 2004). Glander (1981) demonstrated that leaves from unused trees contained condensed tannins. Welker (2004) reported pronounced differential tree use within the tree species, *H. courbaril*, by a group of mantled howler monkeys (the same group used in the present study). Her subjects fed exclusively on the first two to three pairs of young leaves, fresh out of the terminal bud, and only from particular trees during the 1997 and 1998 periods of leaf flush. Observed differential tree use was not related to tree size, degree of accessibility from surrounding trees, phenology (in terms of possessing the utilized plant part), leaf crop size, primary chemistry, and/or tree location in relation to group movements.

Here, we examined whether the previously observed differential tree and leaf stage use in Welker's study group was in response to differences in secondary chemistry and leaf stages within trees. Of interest was not whether the animals completely avoided particular compounds, because *H. courbaril* leaves are fed on, but whether there are maximum tolerance thresholds, either physiological or gustatory, that might explain selectivity within the species. Trees chosen for analysis were (1) differentially fed from during the previous two field seasons (Welker 2004), (2) located in a fairly continuous grove, and (3) predictable in terms of the period of leaf drop and flush with many trees reported to simultaneously possess young leaves during this time (Welker 2004).

Leaves of *H. courbaril* contain three types of secondary compounds, sesquiterpenes (Martin et al. 1972), tannins (Crankshaw and Langenheim 1981; McCloskey 1984), and flavonoids (Lopez 1976; McCloskey 1984; Artavia et al. 1995). Sesquiterpenes are the

primary component of the leaf resin (Martin et al. 1972). Langenheim (1981) believes that the resins/sesquiterpenes are an evolved defense mechanism. Several constituent sesquiterpenes, β-caryophyllene, caryophyllene oxide, α-humulene, humulene epoxide, α-selinene, and β-selinene, in *H. courbaril* have demonstrated herbivore deterrent capabilities (Langenheim et al. 1980; Barnola et al. 1994). Several others, β-cadinene, α-copaene, α-cubebene, and germacrene D, are inducible in response to herbivore damage (Barnola et al. 1994; Agelopoulos et al. 1999; Vrkočová et al. 2000). One of those, α-copaene, plus a variant isomer, δ-cadinene, may play a deterrent role in oviposition rates by two butterfly species (Ling et al. 2003), and δ- and γ-cadinene have suspected bactericidal capabilities (Haznedaroglu et al. 2001). Differential herbivore damage in a congener, *Hymenaea stigonocarpa*, has been attributed to variation in the concentrations of several sesquiterpenes, caryophyllene, humulene, selinenes, γ-cadinene, α-copaene, and α-cubebene (Langenheim et al. 1986). γ-Cadinene is one of the primary components of the defensive secretions of soldier termites of the species, *Constrictotermes cyphergaster* (de Asevedo et al. 2006; Quintana et al. 2003).

Whereas Glander (1981) reported that mantled howler monkeys, at his site of Hacienda La Pacifica, Costa Rica, chose foods that are low in tannins; he believes they are avoiding condensed vs. hydrolyzable tannins. Whereas *H. courbaril* leaves contain condensed tannins (Crankshaw and Langenheim 1981; McCloskey 1984), they are primarily found in the terminal bud where they account for more than 16% dry weight (DW). They decrease rapidly so that by the second pair of young leaves, they account for less than 1% DW (Crankshaw and Langenheim 1981). Glander (1981) states that young *H. courbaril* leaves at his study site, which is in the same region of Costa Rica and also classified as dry forest, do not contain condensed tannins. Langenheim (personal communication) claims that there is little variability in tannin content among trees within a microhabitat. Whereas monkeys may have been avoiding condensed tannins by not consuming the terminal buds, it appears unlikely that condensed tannins played an important role in the observed differential tree use because they occur at such low concentrations in the youngest leaves. Because tannins have not been suspected to play a role in differential tree use, and because there is no research to indicate that those flavonoid compounds found in *H. courbaril* act as feeding deterrents, we examined only sesquiterpene content of leaves in the present study.

Methods and Materials

Study Site The site is characterized as dry tropical deciduous forest (Janzen 1983) and is located in the Santa Rosa Sector of the Area de Conservacion Guanacaste, Costa Rica. Santa Rosa is located 35 km northwest of the town of Liberia, Guanacaste Province, in northwest Costa Rica.

Subjects The focal group was a free-ranging group of mantled howler monkeys. Animals included one male, three females, and one juvenile. All animals were recognizable as individuals in that they were collared or exhibited unique throat morphology or genital pigmentation.

Data Collection Two-minute point time samples (Altmann 1974) were used to record all instances of individual animals feeding on *H. courbaril* leaves. Data were collected during the 1999 period of leaf flush (14/I/99–22/II/99), yielding 136 observations (scans) of *H. courbaril* consumption.

Trees All 22 trees (1) possessed young leaves during the period of data collection, (2) were accessible from surrounding trees (as per Welker 2004), and (3) were located within a fairly

continuous grove that the monkeys passed through every few days during 3 yr of data collection (Welker 2004 and current study). During the 2 yr before the current study, Welker observed the monkeys' differential use of grove trees (Welker 2004). Whereas no data are available for which trees were fed from in 1997, in 1998 the monkeys fed from 9 of the aforementioned 22 trees (Welker 2004). The decision to use only grove trees eliminated the possible confound of the few nongrove trees being located in different microhabitats and thus possibly being exposed to different abiotic conditions.

Weekly phenological data were collected from all focal trees before, during, and after the period of leaf flush. Binoculars were used to estimate the volume of the crown (in 10% increments) made up of (1) leaves at the stage of development that the monkeys were observed to eat, and (2) mature leaves beyond the stage at which the monkeys fed. These two stages will hereafter be referred to as "young" vs. "mature" leaves. These data were used to determine whether trees possessed young leaves and hence were hypothetically "available" to the animals for consumption. Weekly estimates of herbivore damage by insects were collected. These were represented as the total percentage of leaf blade missing (0, <25, 25–50, 50–75, and >75%). Binoculars were used to scan the tree crown for evidence of missing leaf blade area, as evidenced by holes in the leaves. No tree exhibited more than 25% leaf blade loss. Equal numbers of trees exhibited either no damage or less than 25% damage and were thus categorized as "undamaged" ($N=11$) and "damaged" ($N=11$), respectively.

Leaf Specimens Young leaf specimens (3 g/tree) were collected. Specimens approximated the stage of development the animals were observed to eat, i.e., the first, second, and/or third pairs of leaves out of the terminal leaf bud that were ~2 cm in length and very tender. In addition, for six trees that the monkeys fed from, one specimen each of a younger and older leaf stage were collected. The younger stage consisted of 1 g of terminal leaf buds, and the older stage consisted of 1 g of the sixth or seventh pair of leaves out of the leaf bud. The monkeys were not observed to feed on either of these two stages.

All leaf specimens were field-dried in a dry-box by using a light bulb to reduce humidity. Langenheim (personal communication) and Adams (personal communication) recommended the air-drying procedure for field-processing *H. courbaril* samples for subsequent sesquiterpene extraction and analyses. Because sesquiterpenes exhibit only moderate volatility among the terpenoids, Langenheim and Adams have determined that they do not degrade with this technique. Samples were weighed before and after drying.

Laboratory Analyses The sesquiterpenes under examination are contained in the essential oils of the leaf resin. These essential oils were extracted at the Institut für Organische Chemie, Universität Hamburg, Germany. Samples were finely ground in a mortar and pestle with liquid N_2. One gram of ground young leaves was used for each individual tree analysis. Leaf samples were weighed with an Ohaus Analytical Standard Scale, accurate to 0.001 g. Samples were placed in 100 ml of deionized water and hydrodistilled for 3 hr in a Likens–Nickerson apparatus, from boiling point to heat source removal, using *n*-hexane as a collection solvent. Extracts were reduced to uniform volumes under a nitrogen gas jet.

Extracts were analyzed for sesquiterpene content with a Carlo Erba Strumentazione HRGC equipped with a Chrompak CPSil 5 fused silica capillary column (25 m×0.25 mm with a film thickness of 0.25 μm). The oven began at 50°C, after which temperature increased by 3°C/min until 230°C was reached. Hydrogen, with an inlet pressure of 70 kPa, was used as the carrier gas. Chromatograms were recorded with a Merck Hitachi model 2500 with FID electronic integrator. Some compounds were not detected in some specimens, either because they occurred at extremely low concentrations or because they lacked an individual peak.

Because of the resulting truncated data sets (values were missing for some compounds for some specimens), a maximum likelihood estimate (MLE) was substituted at the advice of Dr. Paul Kostyniak (toxicology consultant, SUNY Buffalo, Buffalo, NY, USA).

Mass spectrometric analyses (EI 70 ev) were conducted on a sample of specimens to ensure accurate identification of the gas chromatography peaks. Equipment consisted of a Hewlett-Packard HP 5890 gas chromatograph (CPSil 5 fused silica capillary column, 25 m × 0.25 mm, 0.25 μm film thickness) coupled to a VG Analytical 70-250S mass spectrometer.

All mass spectra were cross-referenced with the sesquiterpene mass spectral library (Joulain and König 1998). In addition, two samples were analyzed independently by Adams by using a Hewlett Packard MSD and his library of essential oils.

Gas chromatography results yielded the relative percentages of component sesquiterpenes in a given sample of essential oil, collectively summing to 100%. Values for individual compounds are relative to one another and not translatable into actual concentrations.

Data Analyses Some sesquiterpenes were combined for data analysis purposes as follows. Each of three oxidized analogs of caryophyllene, i.e., caryophyllene oxides 1, 2, and 3, were examined individually and in combination. In addition, the oxidized analogs were also included with β-caryophyllene, under the heading "total caryophyllene". Whereas caryophyllene oxide occurs naturally in *H. courbaril*, β-caryophyllene is readily converted into these oxidized analogs upon exposure to air (König, personal communication). Thus, some portion of the oxidized forms may have resulted from the rearrangement of β-caryophyllene. Langenheim et al. (1986) grouped caryophyllene and caryophyllene oxide together. α-Humulene and its oxidized analog, humulene epoxide, were also examined separately and together as "total humulene". Finally, α- and β-selinene were examined individually and in combination because Martin et al. (1974) reported that there does not appear to be any significance as to the relative concentrations of the two isomers and there is high variability within *Hymenaea*.

Results

Used vs. Unused Trees Table 1 shows mean relative percentages of the various sesquiterpenes identified in leaf specimens from used and unused trees along with *t*-test results. Specimens from unused trees contained significantly higher values for α-copaene and cyperene. For those trees that were fed from, frequency of use was not correlated with relative percentages of α-copaene ($R=-0.341$, $P=0.304$, $N=11$) or cyperene ($R=0.113$, $P=0.741$, $N=11$). However, when all trees were divided into three categories of use/nonuse (Fig. 1), there were significant or near significant differences between the groups for both α-copaene ($F=3.406$, $df=2$, $P=.054$, $N=22$) and cyperene ($F=3.826$, $df=2$, $P=.040$, $N=22$). Whereas there is an obvious pattern of use associated with α-copaene that may be indicative of a maximum consumption threshold (Fig. 1), cyperene shows no such pattern. The range of values for α-copaene were 0.31–4.16. No feeding scans were recorded for any tree whose leaves had >3% of the compound, and only four scans with >2.5% (three scans for one tree with a value of 2.53% and one scan with 2.98%). Only four unused trees had values of <2.5% vs. seven with values of >2.5%. In the case of cyperene, in all but four trees, values for cyperene were lower in used than unused trees. However, 11 feeding scans were recorded for the tree with the overall second highest relative percentage of cyperene, i.e., 1.74%.

Leaf Stage in Relation to Sesquiterpene Chemistry Young leaves were compared with buds and a more mature stage of development. No significant differences were found between

Table 1 Comparison of mean sesquiterpene values in used and unused trees

Compounds	MLE Values	Mean Score for Used Trees (N=11)	Range for Used Trees	Mean Score for Unused Trees (N=11)	Range for Unused Trees	t Score	P Value
δ-Cadinene		0.80±0.1	0.34–1.38	0.92±0.0	0.47–1.38	0.95	0.356
γ-Cadinene	2	0.47±0.0	0.02–1.02	0.54±0.0	0.35–0.77	0.70	0.499
β-Caryophylline		12.32±1.8	3.29–19.99	7.40±1.5	1.34–16.67	**−2.12**	**0.047**
Caryophyllene oxide 1		1.16±0.1	0.75–1.85	1.48±0.1	0.80–2.22	1.87	0.076
Caryophyllene oxide 2	2	1.53±0.6	0.02–5.63	1.06±0.1	0.45–1.92	−0.78	0.446
Caryophyllene oxide 3		26.06±2.1	15.36–34.75	29.27±2.1	18.48–43.68	1.10	0.283
Total caryophyllene oxide		28.74±1.8	17.76–36.72	31.82±2.1	20.09–46.31	1.10	0.286
Total caryophylline		41.06±2.1	27.92–50.46	39.22±2.3	26.83–49.66	−0.59	0.560
α-Copaene		1.84±0.2	0.31–2.98	2.89±0.3	1.39–4.16	**2.61**	**0.017**
β-Copaene	16	0.08±0.0	0.02–0.28	0.02±0.0	0.02–0.05	−2.11	0.061
α-Cubebene	4	0.18±0.0	0.02–0.40	0.30±0.0	0.02–0.47	1.92	0.069
Cyperene	2	0.55±0.1	0.02–1.74	1.01±0.1	0.57–2.04	**2.30**	**0.033**
β-Elemene		0.91±0.1	0.38–1.70	1.00±0.1	0.30–1.93	0.54	0.597
Germacrene A	7	0.30±0.1	0.02–0.62	0.29±0.1	0.02–1.06	−0.08	0.940
Germacrene D	1	1.76±0.3	0.12–3.42	1.21±0.4	0.02–4.28	−1.10	0.285
α-Guaiene	3	0.35±0.1	0.02–0.82	0.40±0.0	0.15–0.66	0.44	0.670
α-Humulene		2.12±0.3	0.63–3.36	1.32±0.3	0.40–3.13	−2.04	0.055
Humulene epoxide		3.70±0.2	2.65–4.92	4.23±0.3	2.99–6.07	1.61	0.123
Total humulene		5.81±0.2	4.87–7.21	5.55±0.3	4.21–6.86	−0.74	0.466
α-Selinene		2.23±0.4	0.57–4.52	1.38±0.2	0.49–2.51	−2.00	0.066
β-Selinene		4.02±0.6	1.88–7.55	3.14±0.2	2.29–3.82	−1.51	0.146
Selinenes		6.25±0.2	2.52–10.46	4.52±0.3	2.78–6.00	−1.83	0.093
δ-Selinene	1	2.06±0.5	0.02–5.27	2.90±0.3	1.52–4.20	1.44	0.171
Selena 3,7-diene	6	0.16±0.0	0.02–0.29	0.22±0.0	0.02–0.38	1.14	0.266

df=20, significant values are in boldface and numbers of substituted MLE values for missing values are indicated

young and older leaves. However, values for cyperene (t=2.496, df=10, P=0.032) and selena 3,7-diene (t=3.034, df=9, P=0.014) were greater in buds relative to young leaves and those for γ-cadinene were near significant (t=2.348, df=10, P=0.056).

Leaf Damage in Relation to Sesquiterpene Chemistry and Tree Use Undamaged trees contained higher relative percentages of α-copaene, δ-cadinene, and γ-cadinene, and results for α-cubebene are nearly significant (Table 2). Whereas a chi-square test indicated that monkeys did not preferentially feed from undamaged trees (χ^2=1.636, df=1, P=0.201), only four trees categorized as undamaged were fed from vs. seven damaged trees.

Sesquiterpenes with Suspected Deterrent Function A discriminant function analysis was conducted to determine how well relative percentages of α-copaene and cyperene predicted tree

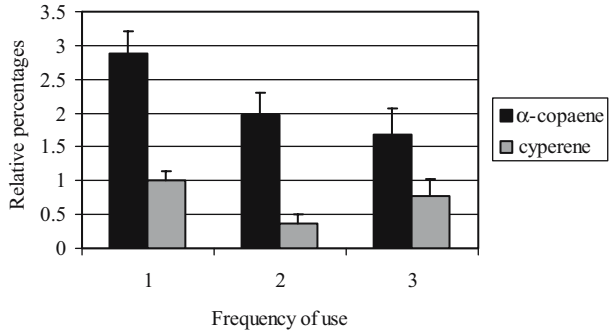


Fig. 1 Relative percentages of α-copaene and cyperene in three categories of use: 1=no scans, 2=1–5 scans, 3=>5 scans

use. Results were significant (Wilks Λ=0.642, χ^2=8.434, *df*=2, *P*=0.015). The discriminant function coefficients were 0.729 for α-copaene and 0.627 for cyperene based upon their degree of correlation with the discriminant function (0.781 and 0.687, respectively). The frequency distribution of the canonical variable for used and unused trees is presented in Fig. 2.

With the exception of α-copaene and γ-cadinene, all compounds implicated in differential insect leaf predation in *H. stigonocarpa* (Langenheim et al. 1986) are intercorrelated in leaf samples from used and unused trees in the present study (see Table 3). As noted, β-cadinene, α-copaene, and α-cubebene have been shown to be inducible in response to herbivorous damage (Barnola et al. 1994). Cyperene was not correlated with any of those compounds.

Discussion

Seventeen sesquiterpenes and several oxidized analogs were identified in young leaf specimens from the 22 study trees. Many are widespread in plant essential oils (δ-cadinene, γ-cadinene, β-caryophyllene, α-copaene, β-copaene, α-cubebene, β-elemene, germacrene D, α-guaiene, α-humulene, α-selinene, and β-selinene) whereas some are known from only one or two genera (cyperene) (Joulain and König 1998). Unused trees contained higher

Table 2 Comparison of mean values in damaged and undamaged trees

Compounds	Mean for Undamaged	Mean for Damaged	*t* Score	*P* Value
Possible deterrent function				
β-Caryophyllene	10.23±2.2	9.49±1.4	0.29	0.773
Total caryophyllene oxides	28.46±1.9	32.15±2.6	−1.16	0.261
α-Humulene	1.70±0.4	1.74±0.2	−0.08	0.936
Humulene epoxide	3.85±0.2	4.08±0.3	−0.67	0.508
α-Selinene	1.89±0.4	1.72±0.3	0.36	0.721
Inducible compounds				
δ-Cadinene	1.00±0.0	0.72±0.0	**2.35**	**0.029**
γ-Cadinene	0.61±0.1	0.40±0.1	**2.46**	**0.023**
α-Copaene	2.87±0.4	1.86±0.2	**2.50**	**0.021**
α-Cubebene	0.30±0.0	0.18±0.0	**2.00**	0.059
Other				
Cyperene	0.79±0.1	0.78±0.2	0.04	0.966

df=20, significant results are in boldface

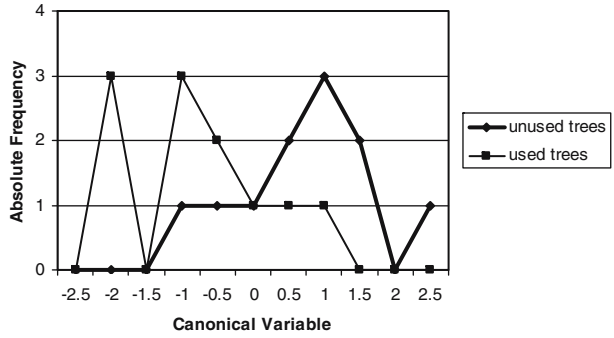


Fig. 2 Absolute frequency distribution of the canonical variable in the discriminant function analysis to determine how well relative percentages of α-copaene and cyperene predicted tree use

relative percentages of α-copaene and cyperene. For those trees that were fed from, no pattern was discernable between relative percentages of cyperene and frequency of tree use. However, in the case of α-copaene, those trees for which few feeding scans were observed (N=1–5 scans/tree) yielded intermediate values when compared with unused trees and more frequently used (N=6–44 scans) trees. These results, along with the relative percentage above which no consumption was observed, suggest the possibility of a dose-dependent effect on tree choice and frequency/duration of use. Whereas young leaves had significantly lower values for cyperene and selena 3,7-diene, when compared with terminal buds, it is difficult to ascertain whether those compounds played a role in bud avoidance as buds contain high concentrations of condensed tannins, and there is evidence of their avoidance by howler monkeys. As mentioned, the occurrence of cyperene is rare relative to other sesquiterpenes, and there are no known reports of a deterrent function. Selena 3,7-diene has not been reported to occur naturally but rather is the result of a rearrangement of β-selinene (Joulain and König 1998). Undamaged trees yielded higher values for α-copaene, δ-cadinene, and γ-cadinene. Finally, α-copaene and cyperene values were found to be predictive of tree use with α-copaene being the strongest predictor.

Langenheim and colleagues have established that populations and subpopulations within *Hymenaea* congeners can be categorized as different chemical "types" based upon the profile of their sesquiterpenes (see, for example, Crankshaw and Langenheim 1981, for a discussion of types). Little intrapopulational variation in sesquiterpene composition, in terms of being classified as one type or another, has been observed in Central American and dry forest *Hymenaea* populations (Martin et al. 1974). Whereas the amount of chemical variation observed in the present study may not be sufficient to qualify them as different

Table 3 Correlation matrix for possible insect deterrent compounds

Compounds	α-Copaene (R)	α-Cubebene (R)	δ-Cadinene (R)	γ-Cadinene (R)	Cyperene (R)
α-Copaene	1				
α-Cubebene	0.642**	1			
δ-Cadinene	0.716**	0.769**	1		
γ-Cadinene	0.233	0.602*	0.650**	1	
Cyperene	0.294	0.019	0.115	−0.012	1

N=22

* P<.01

**P<.001

types, between-tree differences could render leaves from some trees more palatable to the monkeys than others.

There are three reported lines of evidence in support of an antiherbivore function for α-copaene: (1) the facultative production of α-copaene in response to defoliation (Barnola et al. 1994; Vrkočová et al. 2000), (2) differential insect predation in *H. stigonocarpa* trees (Langenheim et al. 1986), and (3) differential oviposition rates by two butterfly species (Ling et al. 2003). Whereas a facultative response is not conclusive evidence of a deterrent function, in that levels of α-copaene likely covary with other compounds that may play a deterrent or maintenance function, the differential leaf consumption by insects and monkeys and differential oviposition rates by butterflies lend support to a possible antiherbivore function for this compound. Interestingly, other compounds that were also implicated in the aforementioned inducibility experiments and differential insect predation and ovaposition rates (i.e., α-cubebene, δ-cadinene, and γ-cadinene) covaried in the present study. Whereas these other compounds did not appear to influence leaf choice by the monkeys, relative percentages were greater in undamaged trees, reinforcing a possible deterrent role for insects.

These results must be treated with caution. Observed differences between young leaf specimens and leaf stages were the result of comparing the relative percentages of compounds in essential oil extracts. Nothing can be concluded about the actual concentrations of those compounds in a given area of leaf blade. A second potential problem arises from the fact that Glander (unpublished data in Glander 1982) reports within-tree variability in leaf quality in relation to location within the canopy for some tree species. Thus, one leaf specimen per tree may not be representative of the average sesquiterpene chemistry of all young leaves in the crown. Whereas, Langenheim and colleagues found little experimental evidence of phenotypic plasticity in resin composition in response to variable abiotic conditions, e.g., light, photoperiod, temperature, and moisture (Stubblebine, unpub.; Langenheim et al. 1975, 1979), there were quantitative differences in resin production and hence the possibility of differing concentrations of the constituent sesquiterpenes.

Results for β-caryophyllene, α-humulene, and α-selinene are of interest in that they have known insect deterrent qualities and yet did not deter the monkeys. Mantled howler monkeys are of moderate size, and considering the relatively high proportion of leaves and flowers in their diet, it seems likely they possess the ability to detoxify a variety of plant secondary compounds.

With regard to leaf stage selectivity, the high concentration of condensed tannins in leaf buds likely precluded their consumption, regardless of sesquiterpene content. Total phenolics and condensed tannins drop from 16% DW to less than 1% DW by the second leaf pair, whereas individual sesquiterpenes may increase or decrease (Crankshaw and Langenheim 1981). Resin yield decreases as leaves become more mature and sesquiterpene and phenolic compounds level off. Mature leaves are tough and, other than some saturniid moth larvae, no herbivores have been observed to feed on them (Janzen 1983). They do not appear to be better defended chemically relative to young leaves. Crankshaw and Langenheim (1981) report that leaf toughness increases between the third leaf pair and the fourth to sixth pairs. Monkeys were not observed to eat leaves beyond the third pair. Whereas Welker (2004) concluded that leaf toughness did not play a role in differential tree use in *H. courbaril*, she considered only toughness of young leaves. Thus, toughness of mature leaves may have played a role in leaf stage selectivity.

In summary, results for α-copaene suggest it as a possible factor affecting palatability and consumption in monkeys. Once the range of concentrations of this compound is determined for this tree species, it must be determined if there is a maximum acceptance

threshold for α-copaene and whether this threshold approximates levels in the leaves of unused trees. With reference to plant part selectivity, the youngest leaves contain lesser amounts of total phenolics, condensed tannins, and certain sequiterpene compounds, relative to the terminal leaf buds. In addition, they are less tough and contain more protein and moisture than mature leaves (Crankshaw and Langenheim 1981). Thus, the animals were feeding on the leaf stage that was of high nutritional value, low in chemical defenses relative to buds, and low in mechanical defenses relative to mature leaves, and appeared to be choosing between individual plants and parts/stages in relation to their primary and secondary chemistry.

Acknowledgement BJW thanks the administration and staff of Sector Santa Rosa for their assistance and permission to conduct fieldwork. Assistance with plant specimen analyses was provided by Paul Kostyniak, Jean Langenheim, Claudia Paul, and Annagret Meiners. Ken Glander and Linda Fedigan provided advice, guidance, and animal tagging. Special thanks to the following field assistants: Bertha Fearon, Graeme Hunt, Alex Perez, and Tanya Smith. Carol Berman provided guidance in the preparation of Welker's dissertation of which this study is a part. Financial support was received from NSF, Sigma Xi, Graduate Student Association of SUNY Buffalo, the Trimble Corp., and various grants from SUNY Geneseo.

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J Chem Ecol (2007) 33:1197–1206
DOI 10.1007/s10886-007-9305-2

Relative Availability of Tannin- and Terpene-containing Foods Affects Food Intake and Preference by Lambs

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Received: 17 August 2006 / Revised: 12 January 2007 / Accepted: 30 April 2007 /
Published online: 11 May 2007
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Abstract The availability of different forages varies across landscapes, but little is known about how proportional consumption of different foods affects food intake and the use of landscapes. The relative amounts of nutrients and plant secondary metabolites (PSM) consumed by herbivores may influence forage intake and animal fitness in landscapes dominated by plants with PSM. Our objective was to determine if the relative availability of nutritious foods that contain PSM affected food preference and intake by lambs. Lambs in three treatments (eight lambs/treatment) were offered two PSM-containing foods at different relative availabilities: treatment 1—*ad libitum* terpene- and 100-g tannin-containing food, treatment 2—*ad libitum* tannin- and 100-g terpene-containing food, and treatment 3—*ad libitum* access to both tannin- and terpene-containing foods. We measured intake of individual foods and total intake of PSM-containing foods during conditioning and a preference test, where animals were offered both PSM-containing foods *ad libitum*. When lambs were fed 100 g of the tannin-containing food, they ingested more terpene and total PSM ($P<0.05$) than when both PSM were offered *ad libitum*, but limiting terpenes did not affect intake of tannin or total intake of PSM ($P>0.10$). During preference tests, all groups preferred tannins over terpenes, but lambs in the treatment fed 100 g of tannin had a higher preference for terpenes than did lambs in the other two treatments ($P<0.10$). These results support the notion that the relative amounts of PSM consumed affects intake and preference for PSM-containing foods by herbivores.

Keywords Relative proportion · Diet mixing · Sheep · Intake · Plant secondary metabolites

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Introduction

All plant species contain plant secondary metabolites (PSM) that limit herbivory and can be toxic at high doses (Cheeke and Schull 1985; Foley et al. 1999). To maintain health, grazing animals must regulate intake of PSM. The variable degree to which herbivores cope with PSM creates differential use of plant species, potentially shifting the balance of competition among plant species in ways that can change ecosystem structure (Council for Agriculture Science and Technology 2002; Villalba et al. 2004). As ecosystems become dominated by invasive plants high in PSM, alternative forages decline because of increased competition and grazing pressure. Ever-decreasing plant diversity further compromises the ability of animals to regulate intake of PSM and meet demands for nutrients (Dearing and Cork 1999; Burritt and Provenza 2000; Villalba et al. 2004), which further increases grazing pressure on palatable plants (Provenza et al. 2003a).

Liver biotransformation and rumen degradation both help herbivores ingest plants with terpenes and tannins. In the liver, PSM are processed through two different detoxification pathways (Freeland and Janzen 1974; Dearing and Cork 1999; Burritt and Provenza 2000; Villalba et al. 2004). In mammals, biotransformation of PSM is carried out in the liver and usually occurs in two steps. The first step (phase I) introduces a reactive group—such as OH, NH_2, COOH, or SH—into the structure of the PSM such that the PSM is made more hydrophilic and polar. During the second step (phase II), the newly formed compound is conjugated with endogenous molecules or groups—glucuronic acid, amino acids, sulphates, or methyl groups—that are hydrophilic (Osweiler et al. 1985). PSM such as terpenes are processed primarily via the phase I pathway, whereas tannins are processed by the phase II pathway (Sipes and Gandolfi 1986). Condensed tannins are large molecules normally not absorbed in the gastrointestinal tract; some are degraded and absorbed to a degree, yielding postabsorptive (potentially toxic) effects (Mehansho et al. 1987; Clausen et al. 1990). Finally, diverse microbial populations in the foregut also are important for PSM biotransformation in ruminants. Terpenes (Johnson et al. 1976; Dizba et al. 2006) and tannins (Foley et al. 1999) both are detoxified by microbes in the rumen.

Previous research suggests that total intake of PSM is higher when tannins and terpenes are fed together rather than when fed in isolation (Villalba et al. 2004; Mote et al. 2007), which suggests that the relative amounts of PSM in a landscape play a key role in how herbivores use forages. Postingestive feedback responses likely are sensitive enough to regulate proportional intake of different PSM (Marsh et al. 2006), as they do with protein and energy (Villalba and Provenza 1999; Provenza and Villalba 2006).

Multiple food alternatives increase sheep intake of foods that contain PSM (Burritt and Provenza 2000; Villalba et al. 2004), perhaps because PSM have different effects in the body and are detoxified by different mechanisms (Freeland and Janzen 1974). Most PSM satiate herbivores (Provenza et al. 2003b); once a threshold of PSM intake is reached, an animal will stop eating the food with that PSM until detoxification is achieved (Foley et al. 1999; Wiggins et al. 2006a, b, c). Satiation on specific PSM in foods may create mild to strong food aversions that decrease subsequent intake and preference for those foods (Provenza 1996). Thus, animals exposed too frequently or to too large a quantity of a specific PSM will satiate on food with that PSM, decreasing their preference relative to an alternative PSM-containing food.

We compared consumption of two PSM-containing foods that varied in availability. We hypothesized that the relative abundance of the two PSM-containing foods—tannins and terpenes—would influence the total amount of food consumed as well as preference for each food. We predicted lambs would decrease their preference for the PSM available

ad libitum and that PSM consumed below satiation thresholds would be preferred, as they were not eaten in amounts high enough to promote aversions.

Methods and Materials

The experiment was conducted with weaned commercial crossbred lambs, averaging 35 kg (SEM=1.09), at the Mote Farm in Willard, UT (NAD 83 UTM 12 T 0413040 4583816). Lambs were individually penned with free access to trace mineralized salt blocks and fresh water. All procedures were approved by the Utah State University Institutional Animal Care and Use Committee.

Test Foods Test foods were formulated to be similar in PSM and nutrient contents to plants found in the sagebrush steppe ecosystem. We used two isocaloric (2.9 Mcal digestible energy/kg dry matter [DM]) and isonitrogenous (126 g crude protein/kg DM; NRC 1985) test foods that contained either tannins or terpenes, similar to those described by Villalba et al. (2004). The tannin-containing food (dry weight) was 76% beet pulp, 9% soybean meal, and 15% quebracho tannin powder. The terpene-containing food (dry weight) was 52.8% beet pulp, 26% grape pomace, 14% soybean meal, and 7.2% of a terpene solution where camphor (18.2 mg/g diet), 1–8 cineole (12.0 mg/g diet), and *p*-cymene (0.7 mg/g diet) were dissolved in 4.1% of vegetable oil. Terpene volatilization was reduced by mixing terpenes with vegetable oil, which also improved terpene homogeneity in the diet (Kimball et al. 1998). All ingredients were ground to 2- to 3-mm particle sizes to facilitate mixing.

Conditioning Twenty-four lambs were stratified by weight and randomly assigned to three treatments (eight lambs/treatment) that differed in the amounts of the tannin- and terpene-containing foods they received: treatment 1—terpene food *ad libitum*, 100 g of the tannin food; treatment 2—tannin food *ad libitum*, 100 g of the terpene food; treatment 3—tannin and terpene foods *ad libitum*. One lamb in treatment 1 was removed from the study because it consistently refused to eat any of the test foods. All lambs were offered both foods simultaneously for 4 hr, beginning at 06:00 each morning, for 12 d. Alfalfa pellets were offered at 10:00 for the first 3 d, at 500, 300, and 150 g $lamb^{-1}$ d^{-1}, respectively, to ensure lambs maintained ruminal health while being introduced to the novel foods. After day 3, all lambs were fed only their respective diets.

Preference Tests After conditioning, all animals were fed alfalfa pellets *ad libitum* from 06:00 to 10:00 for five consecutive days to allow for toxin elimination. The retention time of quebracho tannin in the gastrointestinal tract ranges from 48 (free and soluble) to 72 hr (bound to protein and fiber; Silanikove et al. 1994, 1996). The average primary and terminal elimination half-lives of plasma terpene concentrations is on the order of minutes after terpenes are infused into the rumen or blood stream (Dizba et al. 2006).

We wanted to determine lambs' preferences for tannin- and terpene-containing foods as a function of their prior experience during conditioning, so we offered all lambs a choice between the terpene- and tannin-containing foods for 30 min/d on three consecutive days immediately after the 5-d toxin elimination period. The diet fed during preference tests was the same diet lambs in treatment 3 were fed during conditioning. After preference tests, lambs were fed alfalfa pellets until 10:00, when refusals were collected and no other food was offered until the next day.

Statistical Analyses Data were analyzed as a split-plot design with three treatments (*ad libitum* terpenes, restricted tannins; *ad libitum* tannins, restricted terpenes; *ad libitum* tannins and terpenes) as the whole-plot factor; lambs were nested within treatments. Day was the subplot. Intake of individual foods and total intake of PSM-containing foods were the response variables for both the conditioning and preference trials. Refusals were subtracted from the amount of food offered to calculate grams of food ingested per kilogram body weight (BW). Preference was computed as: [intake of a single-toxin containing food/total intake of toxin-containing foods]×100. Analyses of variance (ANOVAs) were completed by using the MIXED procedure in SAS 9.1 for Windows (SAS, Littell et al. 1996). Data were analyzed with standard procedures for unbalanced ANOVA because of the removal of a lamb from treatment 1. Assumptions of normality and homogeneity of variance were met in normal probability plots and plots of residuals against predicted values.

Results

Conditioning Averaged across the 12 d of conditioning, lambs in treatment 1 that were offered a restricted amount of tannins and *ad libitum* amounts of terpenes ate more terpene-containing food (17.4 g/kg BW; SEM=2.1) than lambs in treatment 3 that were offered both the tannin- and the terpene-containing foods *ad libitum* (5.3 g/kg BW; SEM=2.0; $P<0.05$, Fig. 1). Intake of tannins by lambs in treatment 2 fed *ad libitum* amounts of tannins and restricted amounts of terpenes (8.7 g/kg BW; SEM=1.14) did not differ from that of lambs in treatment 3 (9.4 g/kg BW; SEM=1.14; $P>0.05$, Fig. 1). Averaged across days, lambs in treatment 1 ate more total PSM-containing food (20.2 g/kg BW; SEM=1.7) than lambs in treatments 2 (10.6 g/kg BW; SEM=1.6) or 3 (14.7 g/kg BW; SEM=1.6; treatment effect $P<0.001$; Fig. 1). By day 9, ten lambs had diarrhea—five each in treatments 2 and 3. After lambs were offered alfalfa pellets for 5 d, all signs of diarrhea disappeared.

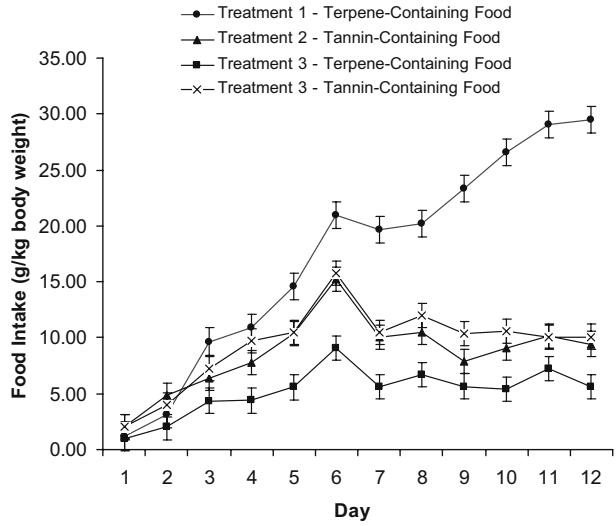


Fig. 1 Daily intake of terpene- and tannin-containing foods by three groups of lambs during 12 d of conditioning. Lambs in treatment 1 (*ad libitum* terpenes, restricted tannins), were offered the terpene-containing food *ad libitum* and only 100 g of tannin-containing food. Lambs in treatment 2 (*ad libitum* tannins, restricted terpenes) were offered the tannin-containing food *ad libitum* and only 100 g of the terpene-containing food. Lambs in treatment 3 were offered both the tannin- and the terpene-containing foods *ad libitum*

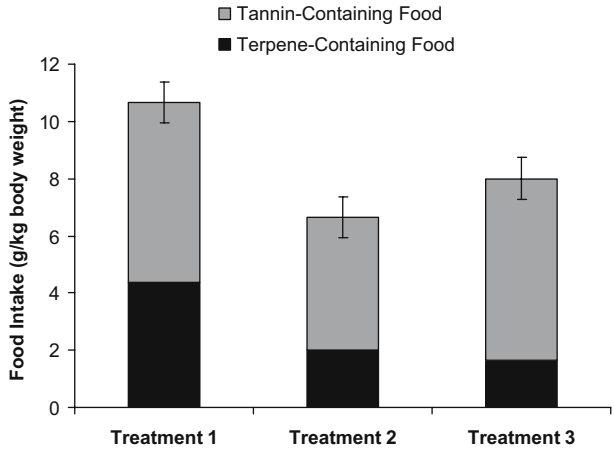


Fig. 2 Intake of terpene- and tannin-containing foods by three groups of lambs during preference tests. During conditioning, lambs in treatment 1 (*ad libitum* terpenes, restricted tannins) were fed the terpene-containing food *ad libitum* and only 100 g of tannin-containing food. Lambs in treatment 2 (*ad libitum* tannins, restricted terpenes) were offered the tannin-containing food *ad libitum* and only 100 g of the terpene-containing food. Lambs in treatment 3 were offered both the tannin- and the terpene-containing foods *ad libitum*

Preference Tests When offered a choice, lambs in all treatments preferred the tannin- to the terpene-containing food, but the differences were less pronounced for treatment 1 (*ad libitum* terpenes, restricted tannins: 59%, $P<0.15$) than for treatments 2 (*ad libitum* tannins, restricted terpenes: 70%, $P<0.10$) and 3 (*ad libitum* tannins and terpenes: 79%, $P<0.05$). Although all lambs preferred tannins to terpenes, lambs in treatment 1 ate more terpene food than lambs in treatments 2 or 3 ($P<0.10$; Fig. 2). Lambs in the three treatments did not differ in intake of the tannin food ($P=0.22$), nor did intake of the terpene food differ for lambs in treatments 2 and 3. Total daily consumption of food with PSM was greater for lambs in treatment 1 (10.7 g/kg BW) than for lambs in treatments 2 (6.7 g/kg BW) or 3 (8.0 g/kg BW; $P=0.002$; Fig. 2).

Discussion

We determined whether the availability of two foods with different PSM affected total food intake and the food preferences of sheep. We hypothesized that the relative amount of tannins and terpenes ingested would influence the amount of food consumed as well as preference for each food. We predicted that lambs would decrease their preference for the PSM available *ad libitum* and that the PSM available in restricted amounts would be more preferred than the PSM available *ad libitum*, as the former were not eaten in amounts high enough to promote satiety and ensuing food aversions (Provenza 1996).

We found that lambs in all treatments preferred the tannin- to the terpene-containing food, but the preference was less pronounced for treatment 1 (restricted tannin, *ad libitum* terpenes) than for treatment 2 (*ad libitum* tannins, restricted terpenes) than for treatment 3 (*ad libitum* tannins and terpenes). The combination of restricted tannins/*ad libitum* terpenes enhanced overall food intake to a greater extent than *ad libitum* tannins/restricted terpenes or *ad libitum* tannins and terpenes. Our results suggest specific flavor-feedback characteristics of the foods, as well as the amount and possibly the order in which they were ingested, influenced intake by lambs of foods that contained tannins and terpenes.

During conditioning, the tannin-containing food induced satiety at a lower level of intake than did the terpene-containing food (Fig. 1). As tannin intake increased relative to

terpene intake, total food consumption decreased indicating an antagonistic relationship between the tannin- and terpene-containing foods. While high intake of tannins (or terpenes) may cause rumen stasis and a general state of anorexia, this was not the case in this study, as lambs in all treatments ate alfalfa pellets readily after conditioning, nor was this the case in previous studies when lambs fed tannin- and terpene-containing foods ate comparable amounts to lambs fed the same nutritious basal foods without these PSM (Villalba et al. 2002a; Shaw et al. 2006a). Rather, Villalba et al. (2004) found that total intake of PSM was higher when tannin- and terpene-containing foods were fed in combination rather than alone. In that study (Villalba et al. 2004), intake of food with terpenes was similar whether terpenes were fed alone or fed with tannins, but the amount of tannin-containing food offered was not restricted; thus, higher tannin intake likely prevented further consumption of terpenes.

In the current study, intake of the terpene-containing food was three times higher when lambs were fed limited tannin and *ad libitum* terpene (treatment 1) than when they were fed *ad libitum* tannin and terpene (treatment 3). Conversely, when terpene was limited and tannin was fed *ad libitum* (treatment 2), tannin consumption was not enhanced relative to *ad libitum* tannin and terpene (treatment 3). Thus, by limiting the availability of the tannin-containing food, tannins were no longer the intake-limiting agent, and the high intake of the terpene-containing food likely was due to a neutralizing effect of a small amount of tannins on the negative effects of terpenes.

Lambs preferred tannins to terpenes, so they likely consumed tannins before terpenes in a meal. If so, tannins in the rumen may have ameliorated the negative effects of terpenes. This hypothesis, while requiring further testing, is consistent with findings of Mote et al. (2007), where tannins and terpenes were fed in different sequences. In that study, the highest PSM consumption occurred with the meal sequence tannin-containing food→ terpene-containing food→nutritious food. Tannins likely bind to small amounts of terpenes reducing their effective concentration or increasing their rate of elimination, allowing sheep to eat more terpene-containing foods. Animals in treatment 3, fed *ad libitum* tannin, likely ingested more tannin than necessary to enhance terpene intake, explaining their lower total intake of PSM-containing food compared with lambs in treatment 1 fed with restricted tannin and *ad libitum* terpene.

Tannins are large molecules (molecular weights from 2,000 to 4,000) that remain in the rumen for many hours (Kumar and Singh 1984; Min and Hart 2003), whereas terpenes are small fat-soluble molecules (Foley and McArthur 1994) absorbed within minutes through the rumen epithelium (Dizba et al. 2006). Tannins are highly reactive molecules that once in the gut are likely to interact with terpenes. Tannins form complexes, mainly through hydrophobic/hydrogen interactions, with a wide variety of compounds including polysaccharides, nucleic acids, alkaloids, and minerals (Okuda et al. 1982; Min and Hart 2003). They also bind to and denature proteins (Jones and Mangan 1977; Burrows and Tyrl 2001). In so doing, they can reduce the production of volatile fatty acids (energy) and the supply of nitrogen (protein) adversely affecting the nutrition of herbivores (Foley et al. 1999).

At appropriate doses, however, tannins can improve the protein status of herbivores, thereby enhancing their ability to consume foods with PSM (Foley and McArthur 1994; Foley et al. 1999; Provenza et al. 2003b). Sheep and goats provided foods high in protein or given a choice between foods high in protein or energy, consume more tannins and terpenes than those fed only with high-energy foods (Villalba et al. 2002a, b). Moderate levels of tannins protect proteins from degrading in the rumen (Barry et al. 2001), which can improve the supply of high-quality amino acids to the abomasum and small intestines (Min and Hart 2003). In our study, tannin- and terpene-containing foods had soybean meal, a rich

source of protein extensively degraded in the rumen (Nocek and Russell 1988). Rumen degradation of protein results in ammonia formation with subsequent loss of nitrogen in the urine (Satter et al. 1999). By binding to the protein in soybean meal, tannins may have protected proteins from degrading in the rumen and improved the supply of high-quality amino acids to the abomasum and small intestines, thus enhancing protein status and increasing terpene consumption by lambs in treatment 1.

Tannins were preferred to terpenes by all groups, but the differences were less pronounced for treatment 1 (59%) than for treatment 2 (70%) than for treatment 3 (79%). Flavor-feedback interactions likely initiated and then maintained the stronger preferences of lambs in treatments 2 and 3 for tannins over terpenes. The flavor of terpenes likely was sufficiently strong to cause lambs to prefer tannins over terpenes in treatments 2 and 3 where lambs were offered tannins *ad libitum*. Conversely, lambs in treatment 1 that were fed limited tannin and *ad libitum* terpene experienced greater exposure to the salient flavor of the terpenes in addition to the postingestive benefit from tannin–terpene interactions. These experiences collectively caused their higher preference for terpenes. This interpretation is consistent with two observations: (1) Several weeks are required for sheep to adapt to the flavor/postingestive consequences of eating foods high in terpenes (Banner et al. 2000) and (2) intake of foods with terpenes increases when alternative foods are restricted (Villalba et al. 2004; Shaw et al. 2006a, b).

Conversely, preference for tannins and avoidance of terpenes during conditioning reduced the ability of lambs in treatments 2 and 3 to maximize food intake. Indeed, their preference for tannins over terpenes caused diarrhea in over half of the lambs in those treatments, whereas no signs of diarrhea were observed in lambs under treatment 1. Fiber digestion in the rumen of sheep is depressed when condensed tannin concentrations reach 4% of DM intake (Roberts and Olson 1999), and tannins (Foley et al. 1999) and terpenes (Nagy and Tengerdy 1968) likely affect rumen bacteria selectively, influencing the ability of herbivores to consume the alternative PSM.

Our present and previous findings (Mote et al. 2007), and those of others (Wiggins et al. 2006a, b, c), suggest that the patchiness of plants with different secondary compounds is important in the foraging behavior of herbivores. Landscapes are mosaics of plant species with varied densities, diversities, and chemistries. Herbivores must move from patch to patch to maintain a balance of macro- and micronutrients. Meanwhile, they must cope with potentially toxic PSM. Preferences for various PSM can change dramatically when PSM are eaten in the right proportions and at the right times. Understanding both the spatial and the temporal contexts that lead to synergy may enable managers of ecological systems to create and maintain mixtures of plant species in a more even manner, creating greater biochemical diversity and reducing the competitive advantage of less-palatable plants that contain high concentrations of toxins.

There is growing appreciation of the importance of interactions between ecosystem chemistry and herbivores (Provenza et al. 2003a). Functional response models predict that food intake is a monotonically increasing function of plant density that asymptotes at maximum intake (Holling 1959). Thus, as the density of a chemotype in a plant community increases, the relative consumption of that chemotype decreases and vice-versa. The proportional disappearance of a plant species through herbivory is inversely related to its density (Provenza et al. 2003a). When the biomass of a chemotype reaches a certain critical amount (escape threshold), that species is increasingly protected from deleterious herbivory, leading to relatively high and ever-increasing use of less-abundant species (Provenza et al. 2003a). Herbivores become quickly satiated on the abundant chemotype and depend upon other complementary chemotypes to maintain appropriate nutrition (Provenza et al. 2003a, b).

The preference for rare species does not necessarily increase, yet they may still be driven to extinction because of their rarity (Provenza et al. 2003a). These processes affect the structure and function of ecosystems (CAST 2002; Villalba et al. 2004), which in turn affect the ability of herbivores to meet their nutritional requirements.

Our study suggests that landscapes high in terpene-containing plants and low in tannin-containing plants may be used differently from ones high in tannins and low in terpenes. Herbivory in a high-terpene/low-tannin system would exert extreme pressure on the tannin components, perhaps to the point of extinction. Assuming plants are equally tolerant of herbivory, at some threshold of plant abundance for tannins and terpenes, as tannins become increasingly rare, the likelihood of tannin-containing plants disappearing from the system increases. A possible synergistic combination could be created in this situation such that herbivores offered limited amounts of tannin could increase pressure on terpene-containing plants helping maintain system stability. The alternative situation (high tannins/low terpenes) would be much more difficult to manage, as terpenes would likely have little influence on the use of tannin-containing plants, and terpene-containing plants may be used to the point of extinction. Thus, extinction rates for terpene-containing plants may be lower than for tannin-containing plants. This hypothesis could be tested with shrubs high in tannins (e.g., bitterbrush, *Purshia tridentata*, and oakbrush *Quercus* spp.) and high in terpenes (e.g., sagebrush, *Artemisia tridentata*). These shrubs codominate much of the sagebrush steppe ecosystem, which occupies nearly 1.1 million km^2 of the western USA and Canada (Meyer and Karasov 1991). Consistent with the proposed interaction between terpenes and tannins, a previous study has shown that sheep manifesting a high preference for sagebrush (terpenes) consume more bitterbrush (tannins) than sheep manifesting a low preferences for sagebrush (Seefeldt 2005).

Finally, our results suggest that tannin-containing plants, such as bitterbrush and oakbrush, may be valuable components of landscapes in the development of grazing systems in the sagebrush steppe ecosystem. By combining the proper proportions of PSM and nutrients, in the right sequence, mangers can potentially optimize the use of dominant plant species with PSM. In our study, a small amount of tannins increased the ability of lambs to consume terpenes, whereas a small amount of terpenes had no such beneficial affect on tannin consumption. Therefore, some dietary mixtures, amounts, and sequences are likely to be more favorable than others nutritionally and physiologically. Thus, we hypothesize that grazing systems that combine limited tannins and large meals of terpenes will more evenly use landscapes high in terpene-containing plants, reducing their competitive advantage and increasing the plant diversity of those landscapes.

Acknowledgements This research was supported by grants from the Utah Agricultural Experiment Station and the Initiative for the Future of Agriculture and Food Systems, USDA (Agreement no. 2001-52103-11215). This paper is published with the approval of the Director, Utah Agricultural Experiment Station, and Utah State University, as paper number 7815.

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J Chem Ecol (2007) 33:1207–1216
DOI 10.1007/s10886-007-9289-y

Chemotaxis of the Pinewood Nematode, *Bursaphelenchus xylophilus*, to Volatiles Associated with Host Pine, *Pinus massoniana*, and its Vector *Monochamus alternatus*

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Received: 20 November 2006 / Revised: 5 February 2007 / Accepted: 13 March 2007 /
Published online: 20 April 2007
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Abstract The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, the most important invasive species in pine forests of Asia, is transported to new pine hosts by beetles of the genus *Monochamus*. Third-stage dispersal juveniles (J_{III}) aggregate in pupal chambers around the vector as it matures. We demonstrated that the ratio of three terpenes (α-pinene, β-pinene, and longifolene at 1:2.7:1.1) released by larval *Monochamus alternatus* strongly attract J_{III}, whereas the different ratio (1:0.1:0.01) of these three terpenes found in healthy xylem of *Pinus massoniana* attracts only the propagative stage (J_n) of the nematode. We suggest that the volatiles produced by the host plants could be the basis of a chemoecological relationship between plant parasitic nematodes and their vector insects. Capture of J_{III} with terpene-baited trap tubes deployed for 2 hr in the field was demonstrated. This technique may lead to the development of rapid sampling methodologies for use at either ports-of-entry or in the field.

Keywords Aggregation · *Bursaphelenchus xylophilus* · Chemo-sensation · Location · *Monochamus alternatus* · Terpenes

Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*(Steiner and Buhrer) Nickle (Tylenchida: Aphenlenchoididae), the causal agent of the destructive pine wilt disease, is native to North America but was introduced to Japan, South Korea, and China, where it has caused irreparable damage to forested ecosystems (Kiyohara and Tokushige 1971; Sun 1982, 2005). It is the most important invasive species in the pine forests of Asia and is

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listed as a quarantine pest in more than 40 countries (Rautapaa 1986; Dwinell 1997; Mota et al. 1999; Schrader and Unger 2003; Yang et al. 2003).

There is no cure for pine wilt disease once a susceptible tree becomes infested with the pinewood nematode. Management of the disease is primarily limited to preventing the introduction and spread of the nematode. The PWN is dispersed to uninfested host pines by its vectors, longhorned beetles in the genus *Monochamus* (Coleoptera: Cerambycidae) (Kobayashi et al. 1984; Linit 1988; Necibi and Linit 1998; Yang et al. 2003; Sun 2005). In Japan and China, *Monochamus alternatus* Hope is the most important vector of *B. xylophilus* (Kobayashi et al. 1984; Sun 2005). Mortality of pine trees caused by the propagative stage (J_n) provides oviposition resources for *Monochamus* (Kobayashi et al. 1984; Linit 1988). As an infested pine dies, the availability and quality of both food and moisture decline, and PWN third-stage juveniles (J_{III}), adapted for dispersal, are produced. These J_{III} aggregate in *Monochamus* pupal chambers as the chambers are formed in the outer xylem by mature *Monochamus* larvae (Mamiya 1972; Linit 1988). In synchrony with beetle eclosion, PWN (J_{III}) molt to the dauer larval stage (J_{IV}) and enter the tracheae of the beetle to be carried to other pine trees (Linit 1988; Necibi and Linit 1998; Yang et al. 2003). The species of *Pinus* attacked, as well as the *Monochamus* spp. utilized as vectors by PWN across its native and invasive ranges, differ (Dwinell 1997; Sousa et al. 2001; Yang et al. 2003). To date, the chemical ecology of *B. xylophilus* in the areas it has invaded has not been studied in detail.

Chemotaxis plays an important role in host location by nematodes during certain phases of their life cycles (Zuckerman and Jansson 1984; Perry 1996). The primary mechanism involves response to chemotactic factors emanating from the host (Zuckerman and Jansson 1984; Grewal et al. 1994; Perry 1996; Zhao et al. 2000). Futai (1980) reported that the J_n of PWN were attracted by the sap of healthy host pines, but they did not identify the chemical basis of the attraction. Chemotaxis by predatory nematodes can occur across a tri-trophic level, through orientation to metabolites given off by plants fed upon by host insects (van Tol et al. 2001; Boff et al. 2002). Maize roots damaged by weevil larvae, *Diabrotica virgifera virgifera* LeConte, release (*E*)-β-caryophyllene, which attracts the animal-parasitic nematode *Heterorhabditis megidis*, thereby bringing the nematodes in close proximity to their prey weevil larvae (Rasmann et al. 2005). In a similar fashion, PWN is transported to new host pines by exploiting chemicals from vector beetles (Miyazaki et al. 1977; Bolla et al. 1989; Necibi and Linit 1998; Stamps and Linit 2001). Effective location of *Monochamus* pupal chambers by third-stage dispersal juveniles (J_{III}) is of primary importance in maintaining the host–vector relationship and allowing exploitation of new host pines (Miyazaki et al. 1977; Bolla et al. 1989). However, species-specific volatile attractants of J_{III} have not yet been identified (Mamiya 1972; Miyazaki et al. 1977; Linit 1988; Bolla et al. 1989; Stamps and Linit 2001).

The objectives of this study were to identify volatiles released by larvae of the vector *M. alternatus* and xylem of the host pine *Pinus massoniana* Lambert, and to determine the effects of these compounds on the chemotaxis of J_{III} and J_n PWN.

Methods and Materials

Insects, Nematodes, and Host Materials In April 2005, fifth instars of *M. alternatus* were obtained from Anhui Province of China. PWNs were obtained from Zhejiang, China. J_{III} were extracted from *M. alternatus* pupal chambers. J_n were cultured with the fungus *Diplodia* sp.

Wood samples of *P. massoniana* were obtained from Anhui, China. Xylem from the walls of *M. alternatus* pupal chambers was collected by drilling to a depth of 10 mm around pupal chambers. Xylem samples containing unidentified species of blue-stain fungi were also collected from regions of dead logs lacking *Monochamus* pupal chambers. Healthy xylem samples were collected from uninfected trees at roughly the same depth as the pupal chambers.

Artificial pupal chambers containing J_{III} were constructed and used for bioassays (Aikawa and Togashi 1997; Jikumaru and Togashi 2003). A hole (4 cm deep and 1-cm diameter) was drilled in the center of the cut ends of healthy *P. massoniana* bolts (5 cm long, 2.5-cm mean diam). The bolts were autoclaved at 121°C for 30 min, then inoculated with *Diplodia* sp. Two weeks later, 3,000 J_n were inoculated into each chamber and the bolts kept at 25°C in the dark for 2 mo.

Chemicals The chemicals used in gas chromatography-mass spectroscopy (GC-MS) analyses and bioassays included various alkanes (Beijing Chemical Plant, >95%), α-pinene (Acros Organics, 98%), β-pinene (Fluka Chemie AG, 80%), longifolene (Acros Organics, 99%), butylated hydroxytoluene (Acros Organics, 99%), and camphor (Acros Organics, 97%). Brilliant Blue R (2%) used to stain the nematode secretions was obtained from Sigma-Aldrich.

Volatile Collection and Analyses To investigate the chemotaxis of J_{III} PWN, we collected volatile samples from both fifth instars of *M. alternatus* and empty *M. alternatus* pupal chambers, as well as from blue-stained and healthy xylem samples of *P. massoniana*, by the Super Q method. Individual *M. alternatus* larvae used for the collection were removed from wood over 2 d. Fresh *P. massoniana* wood samples were harvested immediately before aeration. Samples were aerated in glass vacuum traps (9-cm diam., 100 cm long). A glass pipette containing Super Q (Alltech Associates Inc., Deerfield, IL, USA) was attached to the outlet of the trap. Air, filtered through activated charcoal, was drawn through the apparatus with a QC-1 gas sampler (Beijing Municipal Institute of Labor Protection, Beijing, China) at a rate of 500 ml/min. Samples were aerated for 3 hr. The Super Q cartridges were eluted with *n*-hexane (Fisher Chemicals, 99%), and the resulting extracts stored at −20°C until needed (Lacey et al. 2004).

Volatile collections from either 10 insects or 10 pine samples were combined, dehydrated and concentrated to 250 μl. One microliter of each extract was analyzed by GC-MS with an Agilent 6890N GC coupled to a 5973 mass selective detector. The GC was fitted with a DB5-MS capillary column (0.25 mm i.d.×60 m; Agilent Technologies Inc. Palo Alto, CA, USA), and the column temperature was programmed from an initial temperature of 50°C for 2 min, then increased by 5°C/min to 220°C. Injections were run in the splitless mode. Components of the extracts were identified by comparing retention times and mass spectra with those of authentic standards. Quantification was performed using an internal standard (*cis*-9-hexadecenal) added to each sample (Lacey et al. 2004).

Behavioral Bioassays Two bioassays were used to test the behavioral responses of J_{III} or J_n to volatile chemicals.

Pupal Chamber Bioassay Individual *M. alternatus* larvae, samples of the volatile blend collected from *M. alternatus* larvae by aeration (natural blend), or a synthetic blend of volatiles with the same ratio of terpenes as that collected from the larval aerations, were assayed in artificial pupal chambers. Ten larval equivalents, in 100-μl *n*-hexane, of the

natural or synthetic volatile blends were pipetted onto rubber septa (Tongzhou Shunyi Rubber Co., Beijing, China). Individual larvae, or septa with the natural or synthetic blend, were inserted into the artificial pupal chambers and held at 25°C in the dark for 1 wk. After this, wood chips were collected from walls of each chamber (to about 0.7 cm depth) and from the exterior of each bolt (to a depth of 0.7 cm), and the nematodes present were extracted by using the Baermann funnel technique. The chips were dried to a constant weight for determination of dry weight. An Aggregation Index (AI) was calculated and a one-way ANOVA (using SPSS 11.0 for Windows) used to analyze differences in the AI across treatments.

$$\text{Aggregation Index (AI)} = \frac{\text{Nematodes per g dry} - \text{chips in the interior of chamber}}{\text{Nematodes per g dry} - \text{chips in the exterior of chamber}}$$

Ethylene Sheet-Agar Bioassay The aggregation responses of J_{III} or J_n to volatile collections and blends of monoterpenes were also assayed in Petri dishes (Miyazaki et al. 1977). A volume of warm agar (0.8%, 10 ml) containing 500 nematodes was spread evenly over each plate (9 cm diam.) and allowed to solidify. Ethylene sheets (0.5-cm diam., Reynolds Metals Ltd., Shanghai, China), treated with either single larval equivalents (in 10 µl *n*-hexane) or 10 µl of *n*-hexane alone (control), were used as odor sources. After the solvent had evaporated, one test and one control sheet were placed at opposite ends of each plate with the treated face of the sheet in contact with the agar. The plates were kept in the dark for 24 hr at 25°C. Nematodes aggregating on and under sheets were counted (Miyazaki et al. 1977; Bolla et al. 1989; Stamps and Linit 2001). Differences in the mean numbers of nematodes between treatment and control were compared by Independent-Samples *t* tests.

Field Assays In the field, 50 *P. massoniana* (10–20 cm in diameter) dying from pine wilt disease were sampled from Zhejiang, China, in April 2006. Sampling holes (2 cm deep and 1-cm diam.) were drilled through the entrance holes of *M. alternatus* larvae connected to pupal chambers. Trap tubes were constructed from 2-ml centrifuge tubes (Beijing Zohonice Science & Technology Development Co., Beijing, China) containing a rubber septum inserted into the bottom of the tube. Agar (0.8%) was allowed to solidify around the tube walls and the septa. Volatile blends to be tested were pipetted onto the septa, with control tubes receiving an equivalent volume of *n*-hexane. After the solvent had evaporated, the lids were removed, and the tubes inserted into the sampling holes. Differences in the number of nematodes captured in the treatment and control trap tubes were compared by Independent-Samples *t* test at different times (0.5–48 hr) after commencement of the test.

Effects of Ratios of Three Terpenes on Secretions from J_{III} and J_n The primary chemosensory organs of the nematode are the amphids, which secrete proteins when simulated by molecules of some chemical substances (Perry 1996; Zhao et al. 2000). Secretions of J_{III} and J_n can be detected by staining (Premachandran et al. 1988; Zhao et al. 2000). The respective ratios of the three terpenes found in healthy pines (H3R) and larvae (L3R), were dissolved in dimethyl sulfoxide (DMSO) and distilled water to a concentration of one larval equivalent/10 ul. A 10-µl sample of J_{III} (containing 100 J_{III}) was mixed with 10 µl of 0.2% brilliant blue (in 20% methanol) and 10 µl of one of the terpene solutions or distilled water (control) on top of a microscope slide. The cover slip was sealed with clear nail polish and slides were incubated overnight at ambient temperature in the dark.

Results

In the pupal chamber bioassay, for both the solvent and blank controls (Fig. 1a [S and CK]), more PWN J_{III} were recovered from the wood beyond the walls of the artificial pupal chambers than in the artificial pupal chambers themselves (AI±SE=0.73±0.090). However, the addition of *M. alternatus* larvae to the artificial chamber resulted in the AI increasing to 1.94±0.433 (P=0.048), indicating that nematodes moved into the wood adjacent to the chamber wall. A similar result was also obtained with the volatile extracts from *M. alternatus* larvae (LVE), indicating that the larval volatile extracts contained the components attractive to PWN J_{III} (Fig. 1a). Similarly, in the Petri dish bioassay, the number of J_{III} aggregating on sheets treated with volatile extracts of larvae was significantly higher (P=0.020) than for the control (Fig. 1b). There were no significant differences in the number of J_{III} nematodes on the respective control sheets and on sheets treated with host volatiles collected from healthy, blue-stained, or chamber wood samples of *P. massoniana* (Fig. 1b).

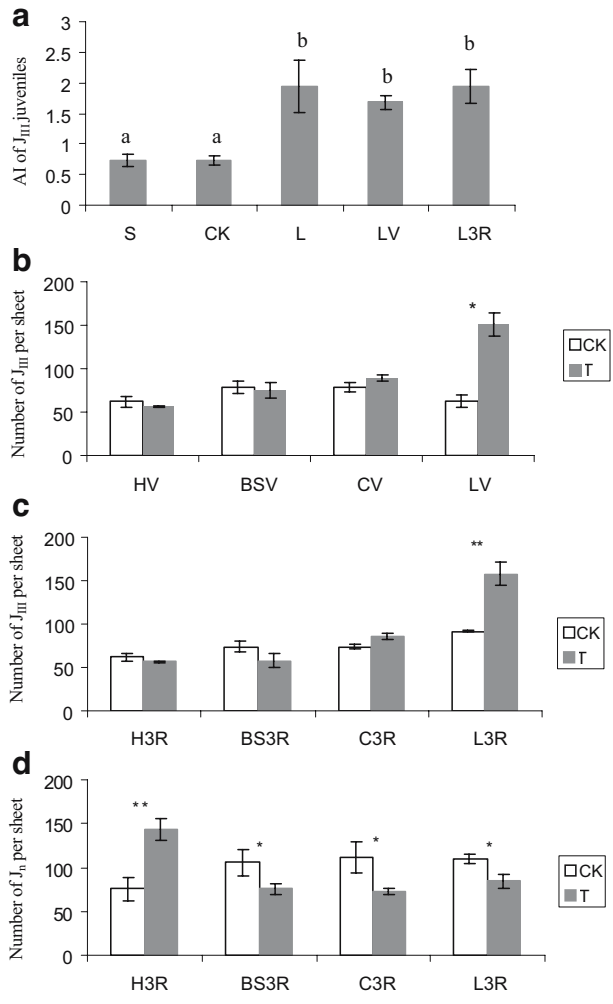


Fig. 1 Aggregation responses of J_{III} and J_n PWN to host and vector volatiles, and blends of terpenes. (**a**) Responses of J_{III} to vector larvae (L), vector larval volatiles (LV) and synthetic terpene blend (L3R) within artificial pupal chambers. AI, Aggregation Index (see text); S, surveyed chambers; CK, control. (**b**) Responses of J_{III} to various volatile collections in Petri dish bioassay. HV, volatiles from healthy pines; BSV, volatiles from blue-stained wood; CV, volatiles from natural pupal chambers. (**c**) Responses of J_{III} to ratios of terpenes in Petri dish bioassay. (**d**) Responses of J_n to ratios of terpenes in Petri dish bioassay. H3R, ratio approximating that of HV=1:0.1:0.01 (1,314 ng/μl); BS3R, ratio approximating that of BSV=1:0.8:0.06 (680 ng/μl); C3R, ratio approximating that of CV=1:0.7:0.08 (242 ng/μl); L3R, the ratio in LV at 1:2.7:1.1 (7.3 ng/μl). Concentrations are expressed for the total amount of the three terpenes. Values indicate the means and standard errors of 10 replicates for each treatment. Bars with the same letters are not significantly different at P>0.05. Asterisks indicate a significant difference from control (*P<0.05; **P<0.01)

The GC-MS traces for the volatile extracts are shown in Fig. 2.Various alkanes (ranging in chain length from C12 to C16) and butylated hydroxytoluene were present only in larval samples, whereas α-pinene, β-pinene, longifolene, camphene, limonene, undecane, and camphor were found in all samples analyzed. Camphor was present in substantially greater amounts in larval samples than in xylem samples. The ratio of α-pinene to β-pinene and longifolene, respectively, varied markedly across the samples. The ratios collected from larvae were lower (1:2.7 and 1:1.1, respectively) than those collected from wood tissue surrounding the pupal chambers or wood from healthy pines (1:0.7 and 1:0.08; 1:0.1 and 1:0.01, respectively).

In Petri dish bioassays, J_{III} of the PWN were not attracted by alkanes, butylated hydroxytoluene, or camphor (data not shown). Differences in the aggregation of J_{III} were noted with four different ratios of three terpenes, α-pinene, β-pinene, and longifolene. Aggregation of J_{III} to sheets treated with a ratio of these terpenes specific to larvae (L3R, 1:2.7:1.1) was significant (P=0.008), whereas ratios of the terpenes matching healthy pines

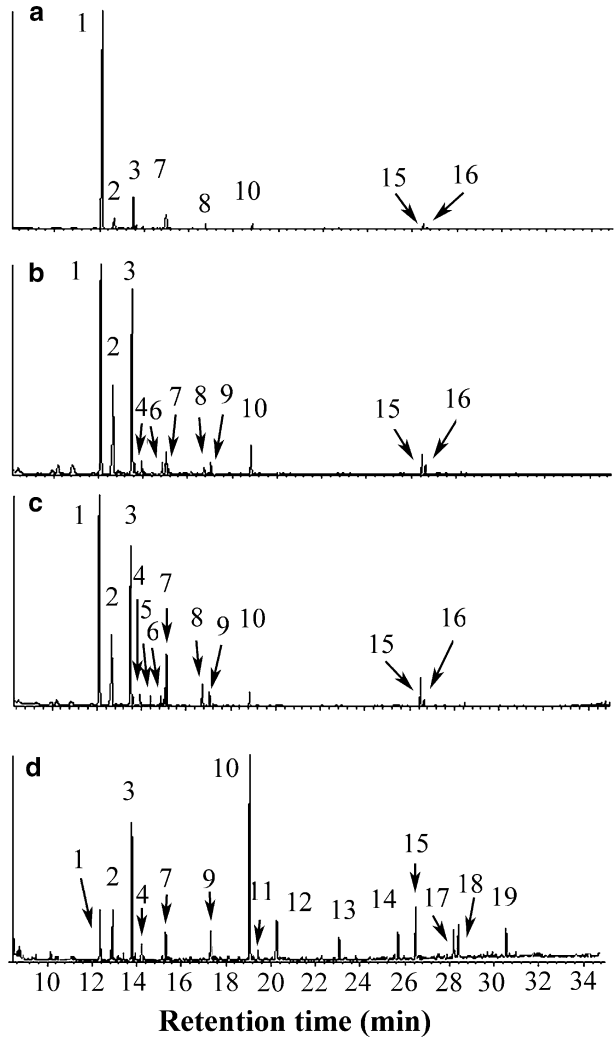


Fig. 2 GC analysis of different volatile collections/treatments. (**a**) Healthy pine samples. (**b**) Blue-stain pine samples. (**c**) Pine chamber samples. (**d**) Larval *M. alternatus*. 1, α-pinene; 2, camphene; 3, β-pinene; 4, decane; 5, 3-carene; 6, *m*-cymene; 7, limonene; 8, β-phellandrene; 9, undecane; 10, camphor; 11, 2-ethylnonane; 12, dodecane; 13, tridecane; 14, tetradecane; 15, longifolene; 16, caryophyliene; 17, pentadecane; 18, butylated hydroxytoluene; 19, hexadecane

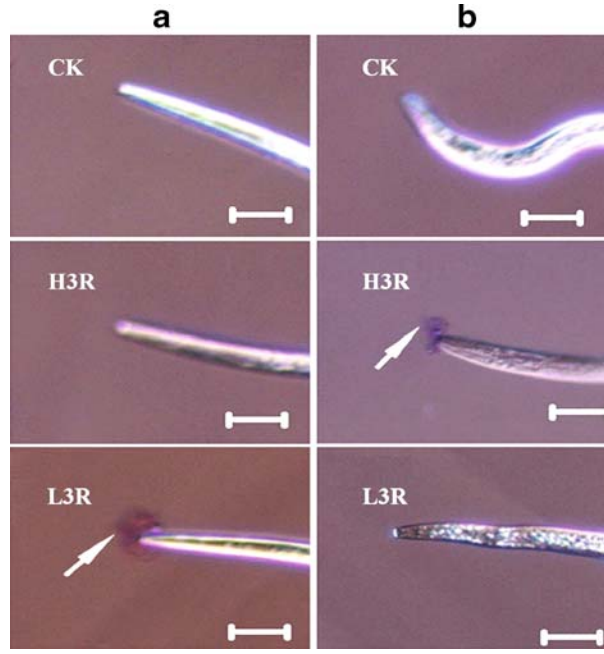


Fig. 3 Stimulation of J_{III} and J_n PWN secretions by different ratios of α-pinene, β-pinene and longifolene. (**a**) J_{III} secretion. (**b**) Stimulation of J_n secretion. Treatments are: H3R, ratio of three terpenes in healthy pine samples at a concentration of 1,314 ng/μl; L3R, ratio of three terpenes released by *M. alternatus* larvae at a concentration of 73 ng/μl. Bar=30 μm. Concentrations are expressed for the total amount of the three terpenes. Arrows point to the stained secretions of nematodes

(H3R, 1:0.1:0.01), blue-stain samples (BS3R, 1:0.8:0.06), and empty pupal chambers (C3R, 1:0.7:0.08) elicited no significant aggregation of J_{III} (Fig. 1c). We also tested the responses of J_n to various ratios of the three terpenes (Fig. 1d). J_n were attracted to the ratio found in natural healthy pine volatiles (H3R, 1:0.1:0.01; P=0.003), but were repelled by BS3R, C3R, and L3R.

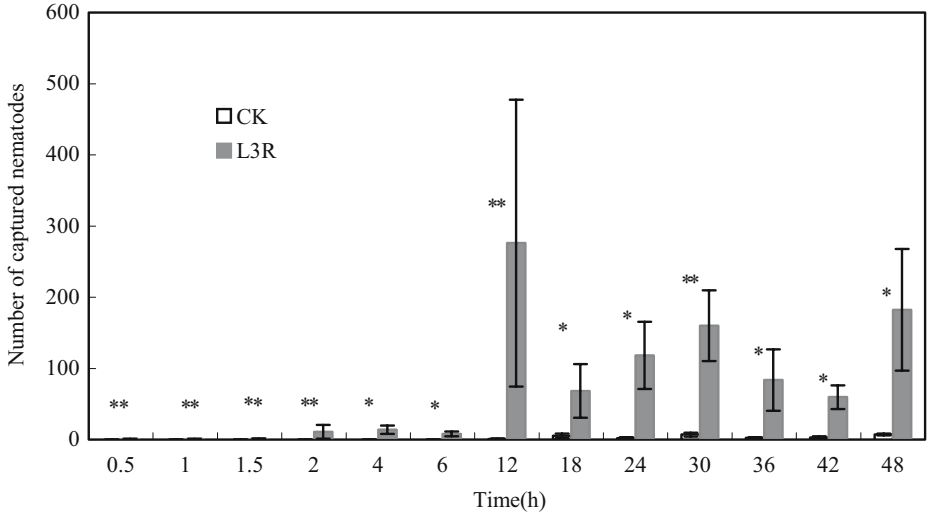


Fig. 4 Catches of PWN in trap tubes, containing different volatile treatments, in the field. Traps were sampled at different times after deployment. L3R, ratio of three terpenes released by *M. alternatus* larvae at a concentration of 7 mg/40 μl. Concentration is for the total amount of the three terpenes. Trial was conducted April 11–15, 2006. Asterisks indicate a significant difference from control (*P<0.05; **P<0.01)

To simulate natural conditions, L3R was also tested in the artificial pupal chamber bioassay. The AI of L3R (1.952±0.277) was significantly (P=0.001) different from that of the control and similar to those of the larvae and larval volatiles (Fig. 1a). This result demonstrates that L3R (1:2.7:1.1) is attractive to J_{III} under natural conditions and that this ratio of the three terpenes is likely the aggregation signal used by the J_{III} juveniles of the PWN.

In the staining assay, for detection of secretions from amphids of PWN, secretions of PWN J_{III} were only detected in response to L3R (Fig. 3a), whereas secretions of J_n were only detected in response to H3R (Fig. 3b). These results demonstrate that the different life stages of PWN distinguish different ratios of terpenes.

In dying *P. massoniana*, significantly greater numbers of nematodes were captured in the L3R-containing traps than in the control traps at all times tested (0.5–48 hr). The greatest number of nematodes was obtained after 12 hr of trapping, with >200 per trap tube baited with L3R (Fig. 4).

Discussion

Animals develop the ability to exploit cues in their habitat during their evolution. We demonstrated that two forms of PWN (reproductive and dispersal) distinguish specific ratios of terpenes produced by their host and vector (Fig. 1; Fig. 3). The reproductive form (J_n) was attracted to the ratio produced by the host tree, but not to the ratio produced by the vector beetle, whereas the dispersal form (J_{III}) was attracted to the ratio produced by the vector, but not to the ratio produced by the host. This attraction demonstrates how J_{III} PWN are able to utilize their vector by moving toward volatile terpenes released by mature *M. alternatus* larvae, allowing the nematodes to aggregate at the site of pupation and adult eclosion of the vector. Subsequent dispersal of the adult beetle allows the nematode access to healthy host pines. Although the specificity and sensitivity of insects to specific ratios of multicomponent pheromones is well established (Monti et al. 1995; Bruce et al. 2005), this is the first report of such a phenomenon in nematodes.

With globalization, biological invasions are a significant environmental threat to the maintenance of natural forest ecosystems (Liebhold et al. 1995). An invasive species must adapt to its new environment. For such a species as PWN, whose life cycle involves multi-trophic interactions, adaptation to its environment can be precarious unless it can utilize both new hosts and new vectors. Our work demonstrates how the alien PWN is able to adapt, by utilizing a common chemoecological relationship between both host and vector, in a new environment.

The PWN is listed as a quarantine target or as a dangerous pest in many countries including those in the European Union (Rautapaa 1986; Dwinell 1997). It has recently been detected in Portugal (Mota et al. 1999). Currently, there is no simple, effective and rapid sampling method available for this pest for use in quarantine monitoring. Our demonstration of chemo-attraction of J_{III} PWN to a specific ratio of terpenes, combined with the successful trapping of PWN, suggests that this approach could be developed into an effective detection method, for use at ports-of-entry and in field detection.

Acknowledgments We thank Daniel Miller, David Kulhavy, Marc Linit, and two anonymous reviewers for critical comments on the manuscript, and Prof. Xingzhong Liu for his technical assistance. We are especially grateful to Leland Humble for his review and editing of the revised manuscript. This study was funded by the CAS Knowledge Innovation Program (KSCX2-YW-N-006), National Natural Science Foundation of China

(30621003, 30525009) and a grant (Chinese IPM0502) from the State Key Laboratory of Integrated Management of Pest Insects and Rodents, IOZ. The publication of this work is also supported by the EU ALARM project. All experiments described in this paper were done according to the rules of the ethical board for animal experiments complying with the current laws of China.

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J Chem Ecol (2007) 33:1217–1225
DOI 10.1007/s10886-007-9293-2

The Effects of a Naturally Produced Benzoquinone on Microbes Common to Flour

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Received: 2 June 2006 / Revised: 12 March 2007 / Accepted: 28 March 2007 /
Published online: 1 May 2007
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Abstract Many species of insects are known to produce and secrete benzoquinone derivatives. These compounds are usually defined as "defense chemicals." However, in many cases, it has not been determined what the chemicals are meant to defend against. Tribolium beetles produce up to three benzoquinones, but their specific function is not known. In this experiment, one of the derivatives, methyl-1,4-benzoquinone (MBQ) was tested for its effectiveness for one of its purported functions as an antimicrobial. Methyl-1,4-benzoquinone was added in three concentrations (0.3, 30, and 150 μg/ml) to the liquid media of three species of *Bacillus* bacteria and eight species from four genera of yeasts, and the effect on their growth was monitored. The presence of MBQ altered growth in all species. The bacteria responded more negatively than the yeasts. All bacteria species showed reduced growth at all levels of MBQ. Yeasts were more tolerant to the presence of the chemical, and two species, *Saccharomyces microellipsoides* and *Pichia burtonii*, actually showed increased growth at the lowest level of MBQ.

Keywords Benzoquinone · *Bacillus* · Yeast · *Pichia* · *Cryptococcus* · *Rhodoturula* · *Saccharomyces* · *Tribolium*

Introduction

Over 100 species of arthropods synthesize benzoquinone derivatives (Blum 1981). Tribolium flour beetles produce up to three benzoquinone derivatives: methyl-1, 4-benzoquinone (MBQ), ethyl-1,4-benzoquinone, and methoxybenzoquinone (Weatherston 1967; Blum 1981). Although often termed "defense chemicals," it is not clear what these compounds actually defend against. Based on potential organismal interactions in their

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stored grain environment, one theory has suggested that the compounds prevent rodents from becoming potential predators on flour beetles (El-Mofty et al. 1992; Dettner 1993; Bouchard et al. 1997). Other hypotheses suggest that the compounds act as antimicrobials (Geiger 1946; Engelhardt et al. 1965; Prendeville and Stevens 2002; Yezerski et al. 2005).

Williams (1966) established a list of criteria for a trait to be considered adaptive. These included widespread examples across species, complexity of the trait, cost of the trait to the organisms, and a well-defined genetic basis. The production of benzoquinones by Tribolium beetles fits these criteria. Benzoquinone derivatives are found in many genera of insects (Blum 1981). There is great complexity in the creation and storage of the compounds, as well as metabolic costs to the organisms. In flour beetles, the compounds are derived from an ill defined, but apparently intricate, mechanism in specialized prothoracic and postabdominal glands (Happ 1968). In addition to the metabolic costs, the substances are known to be harmful to the beetles themselves and thus pose a survival cost (Chapman 1926; Roth and Howland 1941). Further supporting the fact that benzoquinone production is an adaptation, the storage of the benzoquinone derivatives has been shown to have a strong and quantitative genetic background (Yezerski et al. 2000, 2004). However, the exact functions of these chemicals for these beetles has not been clearly defined.

In this research, we explored the hypothesis that the benzoquinone trait is an adaptation in the stored grain pest, *Tribolium confusum*, that controls microbial growth in their flour environment. To this end, we obtained type species of bacteria and yeasts known to be associated with stored flours, and we subjected them to an environment containing varying levels of the benzoquinone derivative, MBQ. The effect of the compound on the growth of the microbial species was monitored via turbidity.

Methods and Materials

Source of Microbial Specimens The three species of bacteria are known to inhabit flour and other stored grain products (Norris et al. 1981). These species were provided in both type strain and flour isolate forms by L. K. Nakamura of the USDA in lyophilized preparations. The species included *Bacillus circulans* [NRRL B-380 (type) and B-393 (isolate)], *Bacillus subtilis* [NRRL NRS-744 (type) and B-571 (isolate)], and *Bacillus licheniformis* [NRRL NRS-1264 (type) and NRS-766 (isolate)]. Specimens were grown and maintained in TGY media containing tryptone, glucose, and yeast extract.

Yeasts were provided in lyophilized form by C. P. Kurtzman of the USDA and included the following species known to inhabit wheat flour (Kurtzman et al. 1970): *Pichia fermentans* (NRRL Y-7138), *Pichia anomala* (NRRL Y-7081), *Pichia burtonii* (NRRL Y-7082), *Pichia farinosa* (NRRL Y-7084), *Saccharomyces microellipsoides* (NRRL Y-6524), *Cryptococcus albidus* (NRRL Y-7079), *Cryptococcus laurentii* (NRRL Y-7139), and *Rhodotorula glutinis* (NRRL Y-7140). Specimens were grown and maintained in YM-2 media containing yeast extract, malt extract, peptone, and glucose.

Inoculation Procedure Measurement of the growth of each species began with inoculation of 1 ml of the microbe that had reached maximum turbidity for the species into 100 ml of the appropriate media in a sidearm flask containing one of three concentrations of MBQ: 0.3, 30, or 150 μg/ml. These values were based on the average amount of MBQ found to be on the external surface of a typical beetle and equated to 1, 100, and 500 beetles, respectively (Yezerski et al. 2000, 2004). A control was simultaneously inoculated similarly into a flask containing no MBQ. All measurements were taken in triplicate. Prior testing by plating

found no inherent microbial growth in the purchased MBQ. Methyl-1, 4-benzoquinone has a strong orange to red color in microbial media, and therefore, "color blanks" were needed that contained the same concentrations as the experimental flasks. However, the presence of the microbes themselves also altered the color of the media as growth occurred and, thus, interfered with the accuracy of the readings. Therefore, blanks were created identical to the control, but cells were centrifuged to the bottom of the tube, removing turbidity, before being used to zero the machine. These blank cultures were then resuspended until the next measurement was taken to allow growth to continue as seen in the controls.

Growth Measurements Turbidity of the inoculations was determined by using a manostat colorimeter. Readings began 6–8 hr after inoculation depending on the previously determined lag time for each species. After the colorimeter was zeroed by using the blanks, readings were taken on the experimental cultures to obtain turbidity in Klett units. This procedure was repeated for each inoculation every hour until the control flasks approached a stationary phase. Notable events between and within species and concentrations were compared via a repeated-measures analysis that used the JMP™ Statistical Program (Sall et al. 2006).

Results

Growth curves for the bacteria species are shown in Fig. 1 and growth curves for the yeast species in Figs. 2 and 3. A summary of a statistical comparison of growth in each condition vs. the control flask is shown in Table 1. The control flasks for all species indicated successful and consistent growth under these conditions. The yeasts tended to reach higher turbidities (around 300 Klett units) than the bacteria, which tended to plateau at levels around 200 Klett units. However, the bacteria reached their stationary phases much sooner than the yeasts under the same conditions.

Compared to the controls, the MBQ-treated flasks showed significantly reduced growth rates in most species (Table 1). The equivalent of the presence of 500 beetles, 150 μg/ml MBQ, appears to have eliminated growth altogether in all of the bacterial species and in three of the eight yeast species. However, considering that 30 μg/ml MBQ was not tolerated by any bacterial species, it is notable that the majority of yeast species was somewhat resistant to the effects of the presence of MBQ even at this highest level. *Pichia anomali* demonstrated little reduction in growth with any level of MBQ, while *B. circulans*' growth was completely eliminated by even 0.3 μg/ml MBQ.

Several of the yeasts appeared unaffected by the compound at the two lowest levels, but their growth was strongly suppressed at the highest level. This is not unexpected because the dilutions were serial and not parallel in nature. However, there is a surprising result for three yeasts, *Rhodotorula glutinus*, *P. burtonii*, and *S. microellipsoides*, where growth increased at the lowest level of 0.3 μg/ml MBQ (Figs. 2 and 3). *Saccharomyces microellipsoides* and *P. burtonii* have significantly higher turbidity than the controls in the second half of their growth curves (Table 1; indicated by Xs rather than asterisks).

In general, the microbial growth was consistently decreased during the measurement time, but bacterial species were more greatly affected than yeast species. There is some indication that low levels of MBQ may actually encourage growth in certain species of yeast.

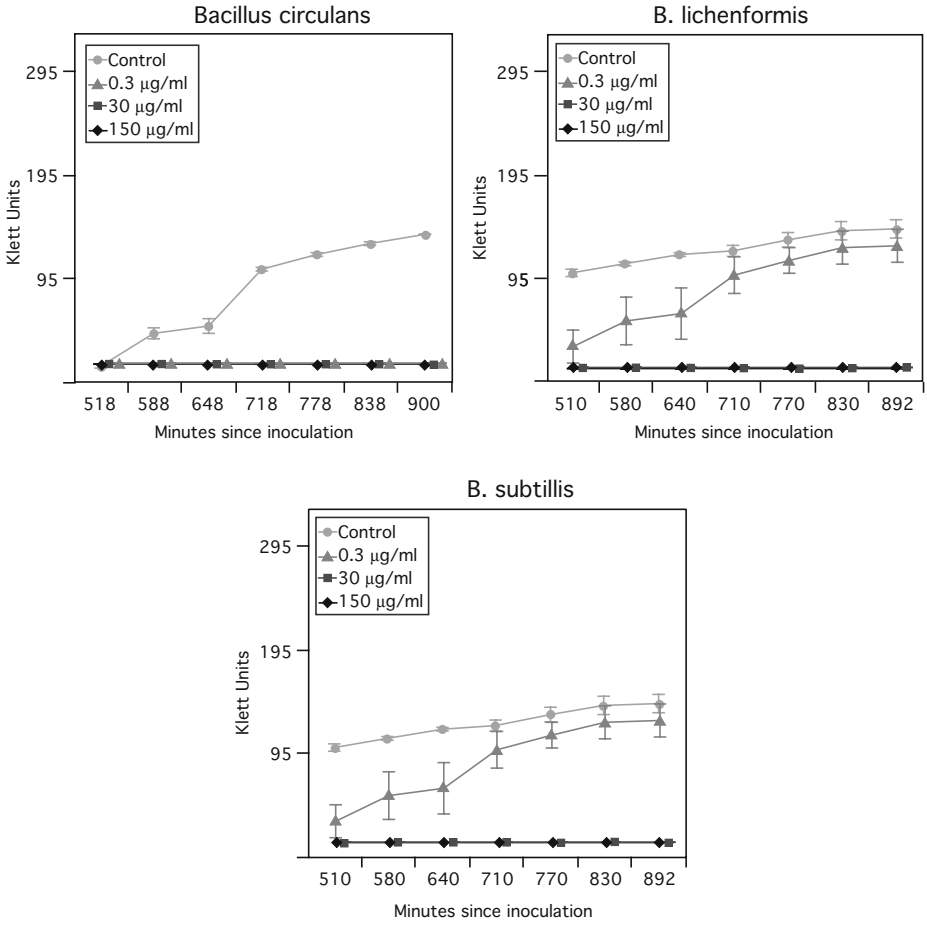


Fig. 1 Growth of three Bacillus species in the presence of MBQ in Klett units + SEM. As indicated in the *legends*, *symbols* represent growth in media without MBQ (*circles*) or with 0.3 µg/ml (*triangles*), 30 µg/ml (*squares*), or 150 µg/ml of MBQ (*diamonds*) measured at the indicated minutes after initial inoculation

Discussion

Our results indicate that MBQ has an effect on microbial growth. However, the compound is more of a deterrent to bacteria than yeast. From an evolutionary standpoint, these results are expected. Bacteria species, especially members of the *Bacillus* genus, can be harmful to insect species by causing numerous diseases and developmental difficulties (Faust 1974; Kreig 1981; Kumari and Neelgund 1985; Abdel-Razek et al. 1999; Abdel-Razek 2002).

Microbes might also compete with beetles for nutritional resources. However, it has been shown that yeast species associated with flour obtain distinct nutritional benefits for the beetles. The beetles do poorly in flour that does not contain sufficient levels of yeast (Offhaus 1952; Charbonneau and Lemonde 1962; Via and Conner 1995). In fact, a veritable symbiosis has been noted (Dunkel 1988). Therefore, the complete elimination of yeast species from the environment would be detrimental to beetle survival.

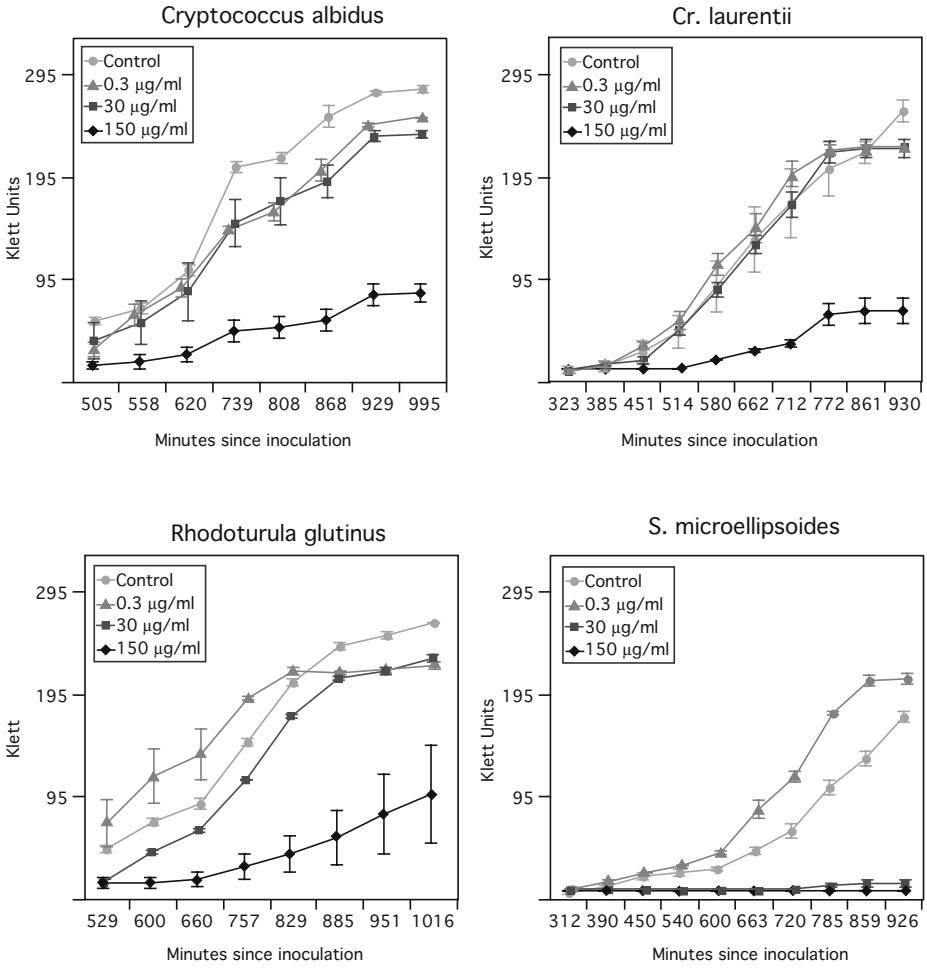


Fig. 2 Growth of four yeast species in the presence of MBQ in Klett units + SEM. As indicated in the *legends*, *symbols* represent growth in media without MBQ (*circles*) or with 0.3 µg/ml (*triangles*), 30 µg/ml (*squares*), or 150 µg/ml of MBQ (*diamonds*) measured at the indicated minutes after initial inoculation

The choice of the bacterial species for this study was based on data for species commonly found in flour according to USDA experts. Species were selected based on previous work specifically with flour isolates. Other sources, however, indicate that, whereas the presence of *Bacillus* species is quite usual, other genera such as *Salmonella* and *Escherichia* should also be considered for future investigation (Farmiloe et al. 1954; Rogers 1978; Okagbue 1990; Richter et al. 1993; Sorokulova et al. 2003). Additionally, our selection of species did not include those that might have been already associated with the beetles themselves because the flour isolates were not from stores containing insects. Recent studies have suggested that insect flour pests introduce members of the Enterococcidae and Enterobacter families into their flour environment, but Tribolium species that produce high levels of benzoquinones generally lack these bacteria species in their stocks (Yezerski et al. 2005). Therefore, although these bacterial species may be

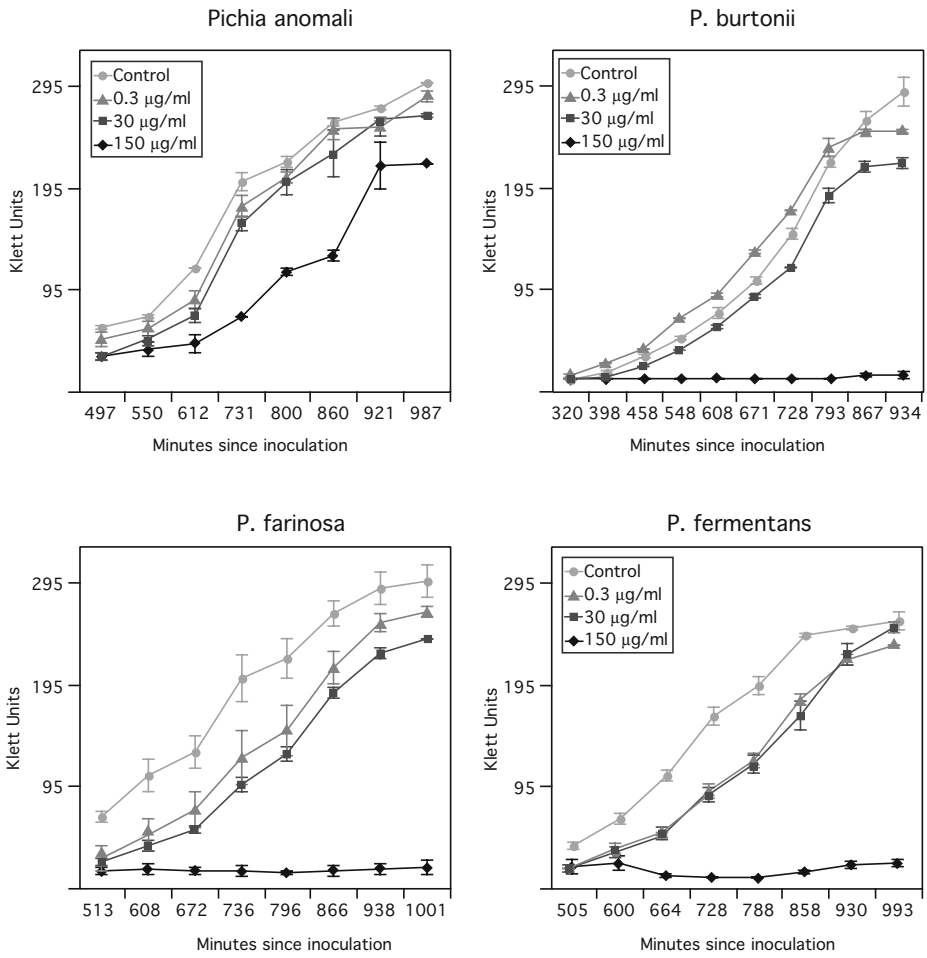


Fig. 3 Growth of four yeast species from the *Pichia* genus in the presence of MBQ in Klett units + SEM. As indicated in the *legends*, *symbols* represent growth in media without MBQ (*circles*) or with 0.3 µg/ml (*triangles*), 30 µg/ml (*squares*), or 150 µg/ml of MBQ (*diamonds*) measured at the indicated minutes after initial inoculation

present in the guts of flour pests, previous work, along with these present data, suggests that the beetles are curtailing their presence.

It is well known that yeasts are associated with flour, but the species varies. The species that we used based on the USDA's flour isolates were not in complete congruence with those suggested by the literature (Kurtzman et al. 1970; Okagbue 1990). However, the species used represent common species. Regardless, the conclusion that yeast species generally grow better in the presence of MBQ than bacterial species is supported.

The levels of MBQ chosen for this study were based on previously determined levels on the external surface of a *T. confusum* beetle (about 0.3 µg per beetle at any given time) (Yezerski et al. 2000, 2004). However, amounts of MBQ on the beetles themselves may differ from amounts present in their flour environment. Other work has shown that beetles secrete approximately 7 µg MBQ over 72 hr, but that much of this degrades into methyl-1,

Table 1 Summary of the statistical comparison of turbidity between control and experimental cultures over time using a repeated measures ANOVA

Species	[MBQ] (μg/ml)	Time Period[a]									
		1	2	3	4	5	6	7	8	9	10
B. circulans	0.3	**	***	***	***	***	***	***			
	30	**	***	***	***	***	***	***			
	150	**	***	***	***	***	***	***			
Bacillus lichenformis	0.3	**	**	**	ND	ND	ND	ND			
	30	***	***	***	***	***	***	***			
	150	***	***	***	***	***	***	***			
Bacillus subtillis	0.3	**	***	***	ND	ND	ND	ND			
	30	***	***	***	***	***	***	***			
	150	***	***	***	***	***	***	***			
C. laurentii	0.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	150	ND	ND	ND	ND	***	***	***	***	***	***
C. albidus	0.3	ND	ND	ND	ND	ND	ND	ND	ND		
	30	ND	ND	ND	ND	ND	ND	ND	ND		
	150	ND	ND	**	***	***	***	***	***		
R. glutinis	0.3	ND	ND	ND	ND	ND	ND	ND	ND		
	30	ND	ND	ND	ND	ND	ND	ND	ND		
	150	ND	ND	ND	**	***	***	***	***		
S. microellipsoides	0.3	ND	ND	ND	ND	xxx	xxx	xxx	xxx	xxx	***
	30	ND	ND	ND	**	***	***	***	***	***	***
	150	ND	ND	ND	**	***	***	***	***	***	***
P. anomali	0.3	ND	ND	ND	ND	ND	ND	ND	ND		
	30	ND	ND	ND	ND	ND	ND	ND	ND		
	150	ND	ND	ND	***	***	***	***	***		
P. burtonii	0.3	ND	ND	ND	xx	xxx	xxx	xxx	ND	***	
	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	
	150	ND	ND	ND	ND	***	***	***	***	***	
P. farinosa	0.3	ND	ND	ND	**	*	ND	ND	ND		
	30	ND	*	**	***	***	**	**	*		
	150	*	**	***	***	***	***	***	***		
P. fermentans	0.3	ND	ND	***	***	***	***	**	ND		
	30	ND	***	***	***	***	***	ND	ND		
	150	**	***	***	***	***	***	***	***		

Blank cells indicate no measurement was taken in this time interval.

ND=no difference

[a] Measurements were taken in 1-hr intervals. The first measurement time from inoculation (1) varied for each species. Actual time in minutes from inoculation can be seen in detail in the accompanying figures. Statistical significance of the comparison of the turbidity in Klett units between indicated culture and respective control. Asterisks represent Klett units significantly lower than control (* $P<0.05$, ** $P<0.01$, and *** $P<0.001$). Xs represent Klett units significantly higher than control (x $P<0.05$, xx $P<0.01$, and xxx $P<0.001$).

4-hydroquinone (MHQ) (Yezerski, unpublished data). It can be assumed that the highly reactive MBQ naturally degrades into MHQ in the flour environment. Therefore, by introducing a reasonable amount of MBQ into the environment and allowing it to degrade normally, one realistically replicates a natural situation. The reactivity of MBQ may be part of the effectiveness of the antimicrobial activity, but this has not been tested directly here.

In this study, we also did not test the chemically related ethyl-1,4-benzoquinone that is also produced by many insect species (Weatherston 1967; Blum 1981). This compound is actually the more abundant compound in the beetle excretion, being produced at a ratio of almost 2:1 in *T. confusum* (Pappas and Morrison 1995; Pappas and Wardrop 1996; Yezerski et al. 2000, 2004). This compound is not commercially available and is also extremely susceptible to light and temperature, making work with it exceedingly difficult (Yamada and Hosaka 1977). The slight structural difference in this substance vs. MBQ perhaps suggests a similar result. The purpose of producing twice as much may be related to ease of natural synthesis.

Although benzoquinone derivatives are a common part of many organisms' physiology, Tribolium beetles and several other insect species incur a metabolic cost that produces a substance that may be harmful to their own survival and development. The results of this experiment indicate that one of these compounds, MBQ, is an effective antimicrobial that is selective to species that are potentially harmful or beneficial. It effectively reduces the growth of bacterial species known to cause harm while controlling and even encouraging the growth of yeast species that are an important food source. Another suggested function may be an antipredatory defense against rats that cohabitate grain storage with flour beetles. We are currently conducting research to determine the efficacy of this alternative hypothesis.

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J Chem Ecol (2007) 33:1226–1234
DOI 10.1007/s10886-007-9288-z

Natural Products Isolated From Species of Halgerda Bergh, 1880 (Mollusca: Nudibranchia) and Their Ecological and Evolutionary Implications

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Received: 12 March 2007 / Revised: 12 March 2007 / Accepted: 19 March 2007 /
Published online: 25 April 2007
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Abstract Chemical investigations of five species of molluscan nudibranchs, *Halgerda*, collected from Australia and Japan were carried out. We identified a novel tryptophan derivative halgerdamine (**1**) along with the known compounds trigonellin (**3**), esmodil (**4**), zooanemonin (**5**), and C2-α-*D*-mannosylpyranosyl-*L*-tryptophan (**2**) from *Halgerda aurantiomaculata*. C2-α-*D*-mannosylpyranosyl-*L*-tryptophan (**2**) was previously thought to be a by-product of tryptophan metabolism exclusive to humans. The only other chordates reported to contain this compound are a number of ascidian species from New Caledonia and Australia including *Atriolum robustum* and *Leptoclinides dubius*. The occurrence of **2** in a mollusc has not been previously reported. Structure elucidation of **1** was achieved by using high-field 2D NMR spectroscopy. No secondary metabolites were detected in extracts from five of the six other species studied, whereas *Halgerda gunnessi* contained mixtures of acylated tetrasaccharides. The compounds isolated from *Halgerda* are different from those found in the close sister taxon, *Asteronotus*. Specifically, species of *Halgerda* contain no terpenes, spirodysin, or bromophenols, as does *Asteronotus*. Furthermore, in contrast to other members of the Nudibranchia such as *Chromodoris* and *Phyllidia*, in which compound yields are quite high, several cryptic species of *Halgerda* seem to lack secondary metabolites, whereas the two highly conspicuous species yield mildly cytotoxic MeOH extracts. Our findings support recent hypotheses regarding progressive evolution of opisthobranchs. In particular, opisthobranchs have evolved to exploit a wider range of

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food and metabolites than did their ancestors, a demonstration of physiological innovation. Some species of *Halgerda* may not be chemically protected either via *de novo* synthesis or by sequestering particularly toxic compounds.

Keywords Halgerda · Asteronotus · Nudibranch defense · Trigonellin · Esmodil · Zooanemonin · Amino acids · Halgerdamine · Tryptophan conjugates · C2-α-*D*-mannosylpyranosyl-*L*-tryptophan · Tryptophan metabolism · Muscarinic agonist · Cytotoxicity · Marine sponges · Evolutionary relationships

Introduction

Nudibranchs have long been recognized as producers of biologically active secondary metabolites. The metabolites are either sequestered from food sources (Cimino et al. 2001) or biosynthesized *de novo* (Cimino et al. 2004). For example, nudibranchs from the family Phyllididae contain sesquiterpenes, isocyanides, and isothiocyanates obtained from the marine sponge *Acanthella* (Dumdei et al. 1997). *Aegires* (= *Notodoris*) *gardeneri* from the family Aegiridae sequesters Clathridine-C, Naamidines E and F that are obtained from *Leucetta* sponges (Carroll et al. 1993). Marine sponge (*Ircinia*) compounds, fasciculatin, variabilin, and palinurin have all been isolated from a member of the family Discodorididae, *Discodoris indecora* (Marin et al. 1997).

The nudibranch family Discodorididae contains 24 genera. Chemical investigations within the family have been rare. Two major clades have been proposed based on morphology (Valdés 2002). Within one clade, only *Asteronotus* and *Peltodoris* have been investigated chemically. *Asteronotus cespitosus* contains brominated phenyl ethers that have been isolated from the sponge *Dysidea herbacea* (Fahey and Garson 2002; Fahey 2003), whereas *Peltodoris atromaculata* contains polyacetylenic compounds isolated from the sponge *Petrosia ficiformis* (Castiello et al. 1980). Within the other major clade, only two genera have been chemically investigated. *Dialula sandiegensis* contains polyacetylenes and isoguanasine (Walker and Faulkner 1981), whereas *Jorunna funebris* contains an isoquinoline alkaloid with antitumor activity (Fontana et al. 2000).

The Doridiidae are the most closely related clade to the Discodorididae (but not one of the two clades that comprise the Discodorididae), and these nudibranchs also have species that sequester compounds such as sterols, nucleosides, alkaloids, unusual and toxic diacylglycerols, and polyacetylenes from their sponge diet (Karuso 1987; Avila 1995; Cimino and Ghiselin 1999; Cimino et al. 1993). The Chromodoridae, a group of cryptobranch dorids that is basally situated to both the Doridiidae and Discodorididae (Valdés 2002), contain mainly terpenes (Karuso 1987). These terpenes are largely the result of a diet of sponges from the Dictyoceratida and Dendroceratida, and notably, the genus *Dysidea*. Members of the Chromodoridae contain isothiocyanates and isocyanides derived from *Axinella* spp., Halichondriidae (Cimino et al. 1999).

Based on these publications, particularly the report that extracts from *Asteronotus* contain sponge compounds (Fahey and Garson 2002), it was of interest to investigate species from other taxa within the clade. *Halgerda*, a sister taxon to *Asteronotus*, has a well-resolved phylogeny (Fahey and Gosliner 2001) but has not been investigated chemically. The genus contains 35 recognized species and represents a major genus within the family Discodorididae. Initial studies with two species (*Halgerda aurantiomaculata* and *Halgerda gunnessi*) indicated that MeOH extract and/or MeOH fractions from C_{18} silica gel separations exhibit weak cytotoxic activity (Fahey 2003). These and other studies have

demonstrated that extracts are antibacterial, cytotoxic, ichthyotoxic, and antifungal (Gunthorpe and Cameron 1987; Avila 1995). The present paper reports on the chemical investigations of five species of *Halgerda* collected from Queensland, Western Australia, and Okinawa, Japan.

Methods and Materials

General Procedures All solvents used were Omnisolv high-performance liquid chromatography (HPLC) grade. High-performance liquid chromatography purification was performed on a Waters Associates model 600 pump with a Waters 996 photodiode array detector with a Betasil C_{18} semipreparative (150×10 mm, 5 μm) column and Betasil C_{18} preparative (150×21.5 mm, 5 μm) column. Optical rotations were measured on a JASCO P-1020 polarimeter (23°C, 10 cm cell). UV spectra were recorded on a CAMSPEC M501 and GBC 916 UV/Vis spectrophotometer and IR spectra were recorded on Nicolet NEXUS FT IR spectrometer. One- and two-dimensional nuclear magnetic resonance (NMR) spectra were recorded on Varian Inova 600- and 500-MHz NMR spectrometers. Samples were dissolved in deuterated dimethylsulfoxide (d_6-DMSO), and chemical shifts were calculated relative to the DMSO solvent peak (^{1}H δ 2.49 and ^{13}C δ 39.5 ppm). Electrospray ionization mass spectra (ESIMS) and high-resolution ESIMS were measured on a Mariner Biospectrometry TOF workstation with positive electrospray ionization, mobile phase 1:1 methanol (MeOH)–water (H_2O) containing 0.1% formic acid.

Collection of Samples Three adult specimens of *H. gunnessi* Fahey and Gosliner (Mollusca: Gastropoda: Opisthobranchia) were collected from one location by using SCUBA on the north side of Rottnest Island, Western Australia (Table 1). Two specimens of *Halgerda theobroma* Fahey & Gosliner were also collected by SCUBA on the north side of Rottnest Island, Western Australia. Five adult specimens of *H. aurantiomaculata* Allan were collected from Heron Island and from Mooloolaba, southeast Queensland; six were collected at Heron Island, Great Barrier Reef; and four were collected at Lady Musgrave Island, Great Barrier Reef. All specimens from Mooloolaba were found on sandy substrate,

Table 1 Nudibranch species collected for the present study

Species	Coloration	Locality[a]	Specimen Storage
H. aurantiomaculata	White ground color, yellow or orange markings	Mooloolaba, SE Queensland	Frozen
		Heron Island, GBR	Frozen
		Lady Musgrave Island, GBR	Frozen
H. gunnessi	Brown ground color, yellow and white markings	Perth, Western Australia	MeOH
H. rubicunda	Red ground color, deep red markings	Zamami Islands, Japan	MeOH
H. theobroma	Brown ground color, yellow markings	Perth, Western Australia	MeOH
H. willeyi	White, pale gray or yellow ground color, brown, yellow, orange markings	Zamami Islands, Japan	MeOH

GBR = Great Barrier Reef

[a] All species were collected either on rubble from coral reefs or sponge gardens or sandy substrate

and not on a sponge. One specimen of each *Halgerda rubicunda* Baba and *Halgerda willeyi* Eliot was collected from the Zamami Islands, Japan.

Extraction and Processing The nudibranch specimens (*H. aurantiomaculata*, *H. willeyi*, *H. gunnessi*, *H. rubicunda*, and *H. theobroma*) were either stored in MeOH or frozen prior to chemical analysis. Specimens of *H. aurantiomaculata* (collected at Mooloolaba) [556 mg dry weight after extraction (DW)], *H. aurantiomaculata* (collected at Heron Island) (2,680 mg DW), *H. aurantiomaculata* (collected at Lady Musgrave Island) (1,360 mg DW), *H. gunnessi* (1,350 mg DW), *H. rubicunda* (60.2 mg DW), *H theobroma* (312.0 mg DW), and *H. willeyi* (210.0 mg DW) were each macerated in MeOH and exhaustively extracted with MeOH, and the extracts evaporated to dryness. Extract masses were: *H. aurantiomaculata* (collected at Mooloolaba), 655 mg; *H. aurantiomaculata* (collected at Heron Island), 1,203 mg; *H. aurantiomaculata* (collected at Lady Musgrave Island), 1,174 mg; *H. gunnessi*, 896 mg; *H. rubicunda*, 22.2 mg; *H theobroma*, 69.6 mg; and *H. willeyi*, 12.7 mg. These extracts were separated by semipreparative C_{18} silica gel HPLC using a gradient from 1% aqueous trifluoroacetic acid (TFA) and MeOH containing 1% TFA over 22 min. One-minute fractions were collected, and fractions were analyzed by (+) ESIMS and ^{1}H NMR. The structures of compounds were assigned based upon 2D NMR analysis or by comparison with literature data.

Results

The weights of nudibranch and yields of extract from *H. rubicunda*, *H. theobroma*, and *H. willeyi* were low, and as a consequence, the NMR analysis of the HPLC fractions was not sensitive enough to detect anything apart from fatty acids and triacyl glycerides (Table 2). The weight of the specimens of *H. gunnessi* (1,350 mg DW) was significantly higher than for the previous three mollusc species, and the MeOH extract and HPLC fractions from this nudibranch were richer in organic components. Fraction 18 from the

Table 2 Compounds identified from the five nudibranchs studied

Compounds	*H. aurantiomaculata*	*H. gunnessi*	*H. rubicunda*	*H. theobroma*	*H. willeyi*
Halgerdamine (**1**)	+	−	−	−	−
Mannosyl-*L*-tryptophan (**2**)	+	−	−	−	−
Trigonellin (**3**)	+	−	−	−	−
Esmodil (**4**)	+	−	−	−	−
Zooanemonin (**5**)	+	−	−	−	−
Tryptophan	+	−	−	−	−
Leucine	+	−	−	−	−
Valine	+	−	−	−	−
Isoleucine	+	−	−	−	−
Fatty acids	+	+	+	+	+
Triacylgylcerides	+	+	+	+	+
Fatty acid esters of acetylated tetrasaccharides	−	+	−	−	−

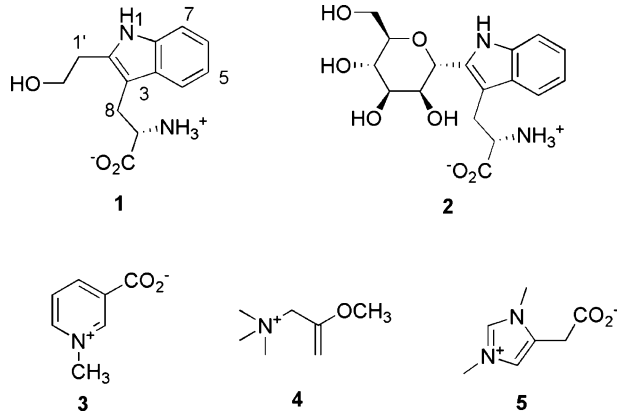


Fig. 1 Compounds isolated from *H. aurantiomaculata* **1** (halgerdamine), **2** (C2-α-*D*-mannosylpyranosyl-*L*-tryptophan), **3** (trigonellin), **4** (esmodil), and **5** (zooanemonin)

HPLC separation contained a mixture of two compounds, which by (+) ESIMS, had molecular weights of 1,455 and 1,469 Da. Further purification by C_{18} silica gel HPLC, isocratic elution with 4:1 MeOH–H_2O did not separate this mixture further, and the yield of compound precluded full structural elucidation. Analysis of gCOSY, gHSQC, and gHMBC spectra suggested that the molecules were long-chain fatty acid esters of acetylated tetra saccharides that also contained an alanine residue. Full structure elucidation will require additional collections of the nudibranchs. High-performance liquid chromatography separation of the MeOH extracts from three different collections of *H. aurantiomaculata* at Heron Island, Mooloolaba, and Lady Musgrave Island yielded the novel tryptophan derivative, halgerdamine (**1**) from the Heron Island collection and the known compounds C2-α-*D*-mannosylpyranosyl-*L*-tryptophan (**2**), trigonellin (**3**), esmodil (**4**), zooanemonin (**5**), and tryptophan from all three collections (Fig. 1, Table 3). These compounds were identified by 2D NMR analysis or comparison with literature data. *Halgerda aurantiomaculata* also contained high concentrations of the amino acids, leucine, isoleucine, and valine.

Halgerdamine (**1**), a novel tryptophan conjugate, was isolated as a colorless gum; $[\alpha]_D^{23}$ + 12.2° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 295 (2.8) nm; and IR (KBr) ν_{max} 1,728, 1,667, 1,590, 1,415, and 1,355 cm^{-1}. Accurate mass measurement of the pseudomolecular ion in the positive HRESIMS at *m/z* 249.1225 (Δ 3.6 ppm) allowed a molecular formula, $C_{13}H_{17}N_2O_3$, to be assigned. The ^1H NMR spectrum of halgerdamine (Table 4) contained signals which could be assigned to a 1,2 disubstituted aromatic ring, a sharp downfield exchangeable proton at δ 10.86, which was either a phenol or an indole proton, a broad

Table 3 Weights (mg) and yields (% dry weight) of compounds isolated from *H. aurantiomaculata*

Collection Location	Halgerdamine (**1**)		Mannosyl-*L*-tryptophan (**2**)		Trigonellin (**3**)		Esmodil (**4**)		Zooanemonin (**5**)		Tryptophan	
	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
Mooloolaba	–	–	0.3	0.05	0.9	0.15	5.5	0.92	1.1	0.18	1.8	0.3
Heron Island	0.3	0.01	1.5	0.06	5.6	0.21	21.0	0.8	3.2	0.12	3.2	0.12
Lady Musgrave Island	–	–	0.9	0.07	3.7	0.29	9.3	0.72	5.4	0.42	2.1	0.16

Table 4 NMR data for halgerdamine (**1**)

Position	$^{13}C^{a}$	^{1}H, mult, *J* (Hz)	HMBC
1	–	10.86	C-2, C-3, C-3a, C-7a
2	136.0 qC	–	
3	104.0 qC	–	
3a	127.2 qC	–	
4	118.1 CH	7.53, d, 7.8	C-3, C-7a, C-6
5	118.5 CH	6.95, t, 7.8	C-3a, C-7
6	120.8 CH	7.05, t, 7.8	C-4, C-7a
7	110.2 CH	7.36 d, 7.8	C-5, C-3a
7a	135.6 qC	–	
8	25.7 CH_2	3.25, m	C-2, C-3, C-3a, C-9, C-10
9	53.2 CH	3.99, m	
10	170.9 qC	–	
1′	28.7 CH_2	2.84, t, 6.0	C2, C3, C2′
2′	60.0 CH_2	3.70, t, 6.0	C2, C1′
NH_3^+	–	8.14, bs	

NMR data are varian unity plus 600 MHz, d_6-DMSO

[a13] C chemical shifts determined from HSQC and HMBC correlations

three proton exchangeable signal at δ 8.14, and five multiplets between 2.84 and 3.99 ppm, two of which integrated to two protons. Analysis of correlations observed in the gCOSY spectrum indicated that the molecule contained an ethyl group and a methylene vicinal to a methine. Halgerdamine was not isolated in sufficient quantity to obtain a ^{13}C NMR spectrum; however, the chemical shifts of all 13 carbons were established from analysis of data obtained from gHSQC and gHMBC spectra. Correlations observed in the gHSQC spectrum suggested that one of the methylenes, C-2' (δ 3.70/60.0 ppm), in the ethyl group was oxygenated, whereas the methine, C-8, was probably attached to an amino group. Correlations from the sharp exchangeable proton at δ 10.86 to four aromatic quaternary carbons, C-2 (136.0 ppm), C-3 (104.0 ppm), C-3a (127.2 ppm), and C-7a (135.6 ppm), supported the presence of an indole. Two of these carbons, C-3a and C-7a, were also long range coupled to the aromatic protons H-4–H-7. The methylene protons H-8a and H-8b also correlated to three of these four aromatic quaternary carbons, C-2, C-3, and C-3a, as well as to a carboxylic acid carbonyl carbon C-10 (170.9 ppm). This indicated that halgerdamine was a tryptophan derivative, which was alkylated at C-2. HMBC correlations from the methylene protons H-1' (δ 2.84) to the quaternary aromatic carbons, C-2 and C-3, and from the oxygenated methylene H-2′ to only C-2 indicated that an ethoxyl group was attached to C-2 of the tryptophan. Although halgerdamine is a simple analog of tryptophan, it has not previously been reported as either a natural product or a synthetic derivative.

Discussion

The observation that *H. aurantiomaculata* contained many polar metabolites and no lipophilic ones is surprising. Most of the compounds previously isolated from nudibranchs have been lipophilic in character. This difference may reflect the isolation techniques used in the past or maybe that *H. aurantiomaculata* is unique in its ability to manufacture or use polar metabolites. Trigonelline (**3**) has previously been isolated from many sources, both

plant and marine. The antibacterial and antineoplastic compound zooanemonin (**5**) has been isolated previously from the marine sponges *Hippospongia equina* (Ackermann and Menssen 1960), *Protophlitaspongtia aga* (Hattori et al. 2001), *Agelas dispar* (Cafieri et al. 1998), *Axinella carteri*, and *Phakellia flabellate* (Carroll, personal communication) and the sea anemone *Anemonia sulcate* (Ackermann and Janka 1953; Ackermann 1954; Ackermann and List 1960). Esmodil (**4**), a muscarinic agonist, has recently been reported from the sponge *Raspailia* sp. (Capon et al. 2004). C2-α-*D*-mannosylpyranosyl-*L*-tryptophan (**2**) was previously thought to be exclusive to humans. Two papers by Garcia et al. (1996) and Kehraus et al. (2004), however, have subsequently shown that this compound is also present in a number of ascidian species from New Caledonia and Australia. The occurrence of **2** in a mollusc has not been previously reported. Compound **2** is known to be a product of tryptophan metabolism in humans. Enzymatic C-mannosylation of tryptophan leads to its detoxification and excretion. The identification of **2** in the tissues of *H. aurantiomaculata* raises the question does *H. aurantiomaculata* feed on an animal that has high levels of C2-α-*D*-mannosylpyranosyl-*L*-tryptophan (**2**) in its tissues, or does the nudibranch have similar enzymatic machinery to humans to detoxify tryptophan by *C*-mannosylation? Furthermore, is C2-α-*D*-mannosylpyranosyl-*L*-tryptophan (**2**) more widely distributed within other phyla and is *C*-mannosylation of tryptophan a ubiquitous detoxification process? The co-isolation of halgerdamine (**1**), a novel tryptophan conjugate, from the Heron Island collection of *H. aurantiomaculata* suggests that the nudibranch possesses enzymes capable of tryptophan metabolism.

A striking finding was the lack of secondary metabolites present in many of the animals studied. This is in contrast to other members of the Nudibranchia such as *Chromodoris* and *Phyllidia* for which compound yields are quite high. For *H. theobroma*, *H. rubicunda*, and *H. willeyi*, no compounds other than fatty acids and triglycerides were observed in yields down to 0.01%. *Halgerda gunnessi* and *H. aurantiomaculata* both gave higher yields of secondary metabolites. It is interesting that low yields of secondary metabolites have been reported from *Asteronotus*. (Fahey and Garson 2002). The presence of secondary metabolites in extracts from *H. aurantiomaculata* and *H. gunnessi* is compatible with the observation that MeOH extracts from *H. aurantiomaculata* and *H. gunnessi* show cytotoxicity (Fahey 2003). Because esmodil (**4**) has previously been isolated from an Australian sponge *Raspailia* (*Raspailia*) sp., *H. aurantiomaculata* may sequester this compound from a dietary source. Furthermore, the observation that *H. aurantiomaculata* contains high concentrations of zooanemonin (**5**), a compound previously isolated from several different sponge sources (*H. equina*, *P. aga*, *A. dispar*, *Phakelia flabellata*, *A. carteri*) suggests that *H. aurantiomaculata* sequesters compounds from a varied sponge diet.

The compounds isolated from species of *Halgerda* are different from those found in the close sister taxon, *Asteronotus*. *Halgerda* spp. contain none of the terpenes, spirodysin, or bromophenols found in *Asteronotus* (Fahey and Garson 2002). Of the species studied, only extracts from *A. cespitosus*, *H. gunnessi*, and *H. aurantiomaculata* were found cytotoxic against P388 cell lines (Fahey 2003). The cytotoxicity of the extracts from the two *Halgerda* species was less pronounced than that of the extracts and compounds isolated from *Asteronotus*. The isolation of high concentrations of esmodil from *H. aurantiomaculata* raises another possible means of protection from predation. Because esmodil is a muscarinic agonist (an acetylcholine mimic), it possesses properties that could cause reactions in predators, such as cardiovascular effects and respiratory depression. Further studies to determine the localization of esmodil and other compounds from *H. aurantiomaculata* within the nudibranch's tissues (in particular the mantle tissue) may identify a defensive role for these compounds.

A question remains regarding the lack of secondary metabolites found in extracts of the three additional species of *Halgerda* examined in the present study. *Halgerda rubicunda*, *H. theobroma*, and *H. willeyi* may prey only upon nontoxic sponge species. Another possibility is that these nudibranchs ingest toxic sponges but have the ability to excrete all toxins before any are sequestered. A third possibility is that these species have evolved the ability to detoxify other compounds that are ingested, similar to the enzymatic machinery noted above for *H. aurantiomaculata*.

Our findings support Cimino and Ghiselin (2001) with regard to the progressive evolution of opisthobranchs. Their discussion suggests that opisthobranchs have evolved to exploit a wider range of food and metabolites, a demonstration of physiological innovation. Basal taxa to the Cryptobranchia (i.e., *Hexabranchus*) appear to consume a rather specific range of food (*Halichondria* sp.), and the more derived clades (i.e., *Hypselodoris* and *Cadlina*) are able to biotransform or biosynthesize selected metabolites for defensive purposes (Cimino and Ghiselin 2001). This study examined one of the most highly derived clades of cryptobranch nudibranchs consisting of *Asteronotus* and *Halgerda*, and our findings suggest that some species of *Halgerda* may not be chemically protected either via *de novo* synthesis or by sequestering particularly toxic compounds. Rather, this group of nudibranchs exploits a wider range of food, including nontoxic sponges, than did its ancestors.

It is interesting to note that MeOH extracts from the two species that are predominantly white in color are toxic and display "warning coloration." The other three species are predominantly dark (brown or dark red) and more camouflaged in their environment and so potentially have less need of chemical protection. Further studies are needed to test this hypothesis.

Acknowledgements The authors thank A. Crowther, S. Grail, S. Millen, and R. Nakano, who collected some of the specimens. J. Hooper, Queensland Museum, identified the sponges. A. Brust, M. Garson, and B. (Stapleton) Dargaville assisted with initial trials of extraction and analyses techniques at the University of Queensland. The late A. Flowers coordinated the cytotoxicity tests performed at Southern Cross University. Funding was provided to SJF by the Australian Federation of University Women through the Freda Bage Award and by the NSF, USA (through PEET grants DEB 99-778155 and 0329054 to T. M. Gosliner). Specimens were collected under permits issued by the Bureau of Fisheries and Aquatic Resources, Philippines (to T. M. Gosliner), the Great Barrier Reef Marine Park Authority (organized by the Queensland Museum), Conservation and Land Management of Western Australia, and the Rottnest Island Authority (organized by the Western Australian Museum).

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J Chem Ecol (2007) 33:1235–1248
DOI 10.1007/s10886-007-9294-1

A Male-produced Sex Pheromone from the Neotropical Redbanded Stink Bug, *Piezodorus guildinii* (W.)

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Received: 8 November 2006 / Revised: 4 March 2007 / Accepted: 13 March 2007 /
Published online: 24 April 2007
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Abstract The male-produced sex pheromone of the neotropical redbanded stink bug, *Piezodorus guildinii* (W.) (Hemiptera: Pentatomidae), a serious pest of soybean in Central and South America, was identified as the sesquiterpene hydrocarbon β-sesquiphellandrene by coupled gas chromatography–mass spectrometry and laboratory bioassays. The identity was confirmed by comparison with an authentic standard, and its absolute configuration was determined to be (7*R*)-(+)-β-sesquiphellandrene. The mean rate of release was approximately 40 ng d^{-1} $male^{-1}$. The compound was found only in extracts from sexually mature males. Laboratory olfactometer bioassays showed that 200 ng of the synthetic compound applied to a filter paper strip was as attractive to female bugs as odors from five 15-d-old live males, an airborne extract from males, or the fraction of the extract that contained (7*R*)-(+)-β-sesquiphellandrene.

Keywords Soybean pest · Sex pheromone · β-Sesquiphellandrene · Olfactometer bioassay

Introduction

The neotropical redbanded stink bug, *Piezodorus guildinii* (W.) (Hemiptera: Pentatomidae), is one of the complex of stink bugs that are serious pests of soybean, *Glycine max* (L.) Merryl, in Central and South America, especially in Brazil (Panizzi and Rossi 1991). More than four million liters of chemical insecticides are used in Brazil each year to control these pests (Corrêa-Ferreira and Moscardi 1996). The use of semiochemical-based methods for control, such as pheromone-based mass trapping or attract-and-kill, would be a more ecologically benign approach to management of these stink bugs.

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The first study of semiochemicals in the genus *Piezodorus* was carried out by Leal et al. (1998). These authors, working with *Piezodorus hybneri* (Gmelin), found in Thailand, Korea, Taiwan, Japan, and some regions of India and Australia (Shepard et al. 1983; Singh et al. 1989), reported that the male-produced pheromone consisted of three compounds, β-sesquiphellandrene, (*R*)-15-hexadecanolide, and methyl (*Z*)-8-hexadecenoate, in the ratio 10:4:1. In laboratory bioassays, both sexes were attracted to this blend.

More recently, Borges et al. (1999) reported the preliminary identification of two possible components of the pheromone blend of *P. guildinii*. Confirmation of these components in the *P. guildinii* pheromone blend and identification of any additional components could provide a species-specific attractant for this species. Thus, the objectives of the study reported here were (1) to collect and analyze the volatiles produced by sexually mature, virgin adults of both sexes of *P. guildinii*; (2) to compare the extracts, looking specifically for sex-specific compounds that might be pheromone components; and (3) to identify, synthesize, and test the biological activity of any such sex-specific compounds.

Methods and Materials

Insects Piezodorus guildinii were obtained from a laboratory colony started from adults collected from soybean fields between 2003 and 2005 near the EMBRAPA Genetic Resources and Biotechnology Laboratory, Brasília, DF, Brazil (15°47′S and 47°55′W). Bugs were reared on raw peanuts (*Arachis hypogaea*), soybeans (*Glycine max*), fresh green beans (*Phaseolus vulgaris*), and water at 26±1°C and 65% relative humidity under a 14:10 light and dark photoperiod (light 06:00 to 20:00 hours). Insects were reared in 8-l plastic containers (100–150/container) with the food supply being renewed three times per week. A 15-cm^2 plastic mesh (~40 mesh) was placed against the inner wall of each container as an oviposition substrate and shelter for the bugs. Egg masses were collected daily and incubated in 9-cm-inner-diameter (ID) plastic petri dishes until they hatched. After the resulting nymphs molted into second instars, they were transferred to plastic containers and reared as above. To prevent interactions between the sexes, males were separated from females after the imaginal molt and cuticular hardening (ca. 24 hr after molting). Sexually mature adults, ≥8 d after the final molt, were used for all bioassays.

Collection of Volatiles Volatiles were collected (N=150 extracts) from groups of 20 male or female *P. guildinii*. The insects were carefully introduced into 1-l glass containers to minimize emission of defensive secretions (Zhang et al. 2003). A vacuum was used to draw air (~1 l/min) into the container through a bed of 4–12 mesh-activated charcoal (Fisher Scientific, Pittsburgh, PA, USA), and out of the container through two traps (15×1.5 cm outer diameter) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL, USA).

Collection of volatiles commenced 5 d after the final molt. Insects were fed with fresh green beans (replaced three times a week) and aerated continuously for 15 d, changing the adsorbent traps daily. The traps were eluted with hexane (4×0.5 ml) and the combined eluates stored at −30°C until used for chemical analysis or behavioral bioassay. To check for background volatiles, the volatiles from green beans alone were collected as described above, over four consecutive days (N=12).

Extracts were concentrated under a gentle stream of N_2 to yield a solution of approximately 0.1 bug-equivalent (BE)/μl, where 1 BE was the amount produced by one male bug in 1 d. For quantitative analysis, an internal standard [25 μg (*E*)-2-octen-1-ol in 50 μl of hexane] was added to the solution.

To evaluate the circadian rhythm of pheromone release, volatiles were collected and quantified during three different periods: morning, from 07:00 hours to noon (N=7); afternoon, noon to 17:00 hours (N=5); and evening, 17:00 to 22:00 hours (N=5).

Analysis and Fractionation of Extracts Crude extracts and fractions thereof were analyzed on a PerkinElmer gas chromatograph (GC; DB-1 column, 30 m×0.25 mm ID, 0.25 μm film; J&W Scientific, Folsom, CA, USA) equipped with a flame ionization detector (FID), using a temperature program of 50°C (for 2 min) increased by 15°C/min to 250°C. Crude extracts were also analyzed at the University of California, Riverside, with a Hewlett-Packard 6890 GC interfaced to an HP-5973 mass selective detector, with electron impact ionization (70 eV). A DB-5 MS column was used (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific), with a temperature program of 40°C (for 1 min), 10°C/min to 250°C, injector 250°C, and transfer line 280°C. Injections were made in splitless mode.

An extract was fractionated as described by Leal et al. (1998). A column (6.0 cm× 0.5 cm ID) containing 500 mg of silica gel (80/100 mesh), impregnated by 10% with weight $AgNO_3$ (Merck, Darmstadt, Germany), was conditioned by eluting with 6 ml of 25% ether in hexane, followed by 6 ml of hexane. Three 24-hr aeration extracts from 20 mature males in ~1 ml pentane were loaded onto the column, and eluted sequentially with 3×1 ml pentane, 3×1 ml 5% ether in pentane, 3×1 ml 10% ether in pentane, and 3×1 ml 100% ether, collecting each aliquot as a separate fraction. The first two fractions of 10% ether in pentane, containing primarily β-sesquiphellandrene, were combined (fraction A). All the remaining fractions were recombined into one solution (fraction B) for bioassay.

Components in extracts from both sexes of *P. guildinii* were tentatively identified by comparison of mass spectral data with library data (NIST-Wiley database, 2000). Identifications were confirmed by comparison of retention times and mass spectra of insect-produced compounds with those of authentic standards.

Determination of the Absolute Configuration of β-Sesquiphellandrene The determination of the absolute configuration of the *P. guildinii* male-specific sesquiterpene, β-sesquiphellandrene, was carried out as described by McBrien et al. (2002) (Fig. 1). The enantiomers of β-sesquiphellandrene were not resolved on a Cyclodex B chiral stationary phase GC column (J&W Scientific), but the column did resolve the enantiomers of the related compound, α-curcumene. Consequently, an aliquot of extract (containing β-sesquiphellandrene) was dehydrogenated to α-curcumene (Jackman 1960) by treatment with 100 μl of a benzene solution of 1,2,3,4-tetrachlorobenzoquinone (20 mg/ml) in a sealed vial at 80°C for 1 hr. The cooled reaction mixture was filtered through a 1-cm plug of silica gel, eluted with pentane, and the eluate analyzed with a Cyclodex B column (30 m×0.25 mm ID) operated isothermally at 90°C (140 kPa helium head pressure). Authentic samples of (+)- and (-)-β-sesquiphellandrene (see below) were dehydrogenated in identical fashion to provide standards for comparison.

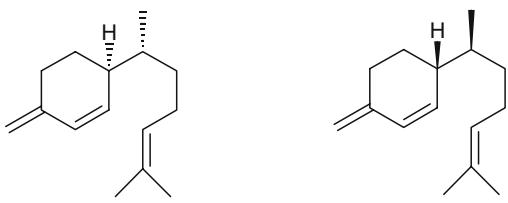


Fig. 1 Enantiomers of sesquiphellandrene. (7*R*)-(+)-β-Sesquiphellandrene is produced by male *P. guildinii*

A second aliquot of the insect-produced β-sesquiphellandrene was hydrogenated by stirring it with 5% Pd on carbon catalyst under H_2 for 1 hr. This produced two diastereomers in a ratio of ~2.5:1. Samples of synthetic β-sesquiphellandrene enantiomers (see below) and a sample of (*S*)-β-sesquiphellandrene isolated from ginger oil (McBrien et al. 2002) were hydrogenated in identical fashion to provide standards. All samples were analyzed isothermally (75°C) on the Cyclodex B column.

Olfactometer Bioassays A two-choice olfactometer modified from Borges and Aldrich (1994) was used to test the biological activity of odors from live insects, aeration extracts of male *P. guildinii*, and fractions thereof. The olfactometer consisted of a 500-ml three-neck, round-bottom flask (24/40 joints, Kontes, Vineland, NJ, USA). Two 250-ml rotary evaporator trap adapters (24/40 joints) were attached to the two outside arms to provide treatment and control chambers. A charcoal filter (20/40 mesh, 130×10-mm ID), connected in series to a water bubbler to humidify the incoming air, was attached to the inlets of the two chambers with silicon rubber tubing (Silastic, 4.8 mm ID; VWR Scientific, Darmstadt, Germany). Air was pulled through the system (400 ml/min) by connecting the middle neck of the flask to a regulated vacuum source, with the flow measured with a Clear Flow Rotameter (Accura Flow Products, Warminster, PA, USA). The olfactometer was positioned horizontally on a countertop in a room with fluorescent lighting (4×40 W). Temperature in the room was maintained at 26.0± 1.0°C. Positions of control and treatment arms were alternated between replicates to avoid any positional bias. The apparatus was cleaned after each five replicates with fragrance-free liquid soap, rinsed thoroughly with water, and dried in a convection oven at 80°C.

To begin an assay, a single *P. guildinii* was gently introduced into the release chamber with an artist's paint brush (Camel Hair, number 1), and allowed to acclimate for 3 min while the remainder of the apparatus was assembled. After attaching the treatment and control chambers and starting the airflow, the test bug's behavior was recorded over 15 min. We recorded the first arm of the olfactometer that each insect chose and subsequently remained in for at least 100 sec. Insects that did not leave the release chamber of the olfactometer during the first 10 min were considered "nonresponders," and were not included in the data analyses.

To determine which sex produced pheromone, we tested the attraction of insects of both sexes to odor from males (N=51 for females and N=30 for males) or females (N=54 for females and N=32 for males). Test individuals were offered a choice between odor from five live, sexually mature virgin insects or a clean air control. The five live insects used as odor sources were changed after 10 replicates.

Bioassays using crude extracts from males were performed with females (N=74) or males (N=30). Five microliters (0.5 BE) of crude hexane extract of the volatiles produced by 20 *P. guildinii* males aerated for 24 hr were spotted on a strip of filter paper, and the paper was placed in one of arm of the olfactometer. Extracts used in these tests contained 800±300 (N=8) ng/μl of β-sesquiphellandrene (ca. 40 ng μl^{-1} $insect^{-1}$ day^{-1}). A control, consisting of a filter paper treated with hexane was placed in the other arm.

To determine when females were most active and responsive to pheromone, bioassays were performed during three periods: morning (07:00 hours to noon) (N=23), afternoon (noon to 17:00 hours) (N=36), and evening (17:00 hours to 22:00 hours) (N=64). For these bioassays the methodology described above was followed.

To test the biological activity of crude extracts of males, fractions of extracts, and synthetic standards, and solutions of test stimuli (1 BE) were spotted on a strip of filter paper (1.5 cm long, 0.5 cm wide), and placed in one of the arms of the bioassay apparatus. Controls, consisting of filter papers treated with hexane, were placed in the other arm. Two

hundred nanograms of each synthetic standard were used in the bioassays. Bioassays were carried out during the evening period, using virgin, sexually mature females.

Fraction A, containing primarily β-sesquiphellandrene (N=70), and fraction B, containing the other volatiles released by male *P. guildinii* (N=34), were tested against hexane controls. Fractions A and B were also directly compared (N=30), as was fraction A against crude extract (N=30) or (7*S*)-(−)-β-sesquiphellandrene (N=30) isolated from ginger oil. Synthetic (7*R*)-(+) and (7*S*)-(−)-β-sesquiphellandrenes were tested against *n*-hexane controls (N=55 and N=54, respectively). In further tests, females were tested with the following comparisons: fraction A vs. synthetic (7*R*)-(+)-β-sesquiphellandrene (N=39) or synthetic (7*S*)-(−)-β-sesquiphellandrene (N=40); (7*R*)-(+) vs. (7*S*)-(−)-β-sesquiphellandrene (N=80); and a 1:1 mixture of synthetic (7*R*)-(+) and (7*S*)-(−)-β-sesquiphellandrenes vs. each enantiomer individually [N=72 for (*R*) and N=65 for (*S*)]. In addition, the biological activities of the last treatments were evaluated in bioassays with males (N=27 and N=34, respectively).

Data on the responses of males and females to different treatments (live insects, aeration extracts and fractions thereof, and synthetic standards vs. controls) were analyzed with chi-square tests. Statistical analyses were carried out by using MathCAD 2001 (MathSoft). Data on the production of pheromone during different periods of the day were analyzed with repeated measures ANOVA (Sigma Stat 3.1 Systat Software Inc.).

Chemicals The synthetic sesquiphellandrenes were synthesized from (*R*)-(+)-citronellal and (*S*)-(−)-citronellal, respectively (Aldrich Chemical, Co., Milwaukee, WI, USA), by the method of Chavan et al. (1997). Each consisted of an inseparable, ~1:1 mixture of the resulting sesquiphellandrene enantiomer with its C6 ring-junction epimer. The enantiomeric purity of the (7*R*)-isomer was 84% enantiomeric excess (ee) (reflecting the enantiomeric purity of the citronellal starting material), whereas the (7*S*)-enantiomer had an optical purity of >98%. In addition, enantiomerically pure (7*S*)-sesquiphellandrene was isolated from ginger oil as previously described (Millar 1998). β-Bisabolene and α-curcumene were available from previous studies (McBrien et al. 2002), (*E*)-β-farnesene was purchased from Bedoukian Research (Danbury, CT, USA), and nonanal was obtained from Avocado Research (Heysham, UK). Samples of *cis*- and *trans*-β-ocimene were kindly provided by Peter Landolt (US Department of Agriculture, Yakima, WA, USA). All other authentic standards were purchased from Aldrich.

Results

Analyses of Extracts of Volatiles Comparison of gas chromatograms and mass spectra of volatile compounds collected from sexually mature virgin males and females showed a number of compounds in common (Table 1). Most compounds had been reported previously from other stink bug species (Borges and Aldrich 1992; Zarbin et al. 2000), including (*E*)-2-hexenal, (*E*)- and (*Z*)-β-ocimene, 2-ethylhexanol, (*E*)-2-hexenyl acetate, undecane, nonanal, dodecane, tridecene, and tridecane. In addition, the extracts contained variable amounts of a group of compounds that were tentatively identified as sesquiterpenes, based on their molecular ions (*m/z* 204 or 202); the general profiles of their mass spectra, which contained patterns of ions typical of sesquiterpene hydrocarbons (Joulain and Konig 1998); and their GC retention times on a nonpolar DB5-MS column, which fell between those of tetradecane and hexadecane. These compounds were tentatively identified as (*E*)-β-farnesene, α-curcumene, zingiberene, β-bisabolene, and the male specific compound β-sesquiphellandrene by mass spectral matches with database spectra.

Table 1 Compounds identified in volatiles collected from sexually mature virgin adult *P. guildinii*

Compound	Amount (%, Mean±SE) Extract from Volatile Collections[a]	
	Male (*N*=4)	Female (*N*=3)
(*E*)-2-Hexenal	13.0±5.3	1.9±0.8
α-Pinene	20.6±6.3	5.9±1.2
Ocimene	0.6±0.3	4.8±1.8
(*E*)-2-Hexenyl acetate	1.4±0.7	15.1±1.5
2-Ethylhexanol	0.3±0.1	3.6±2.2
Undecane	2.6±0.5	4.4±1.9
Nonanal	0.7±0.4	3.8±2.3
Dodecane	2.2±1.5	39.4±13.8
Indole	0.3±0.2	6.2±1.4
Tridecene	0.7±0.1	13.5±6.8
Tridecane	100	100
(*E*)-β-farnesene	29.9±11.9	6.2±5.9
α-Curcumene	3.5±2.4	0.10±0.05
Zingiberene	3.3±3.1	0.10±0.03
β-Bisabolene	5.4±3.5	0.20±0.01
β-Sesquiphellandrene	94.3±46.3	0±0

[a] The mean percentages of compounds relative to tridecane, individually produced by each sex, were obtained from volatiles collection from the first day of collection, where the insects were stressed by manipulation.

The identifications were confirmed by mass spectral and retention time matches with those of authentic standards. All these compounds were of insect origin because they were not present in control aerations of the green beans used as food during aerations (Borges et al. 2006).

Despite the care taken in loading stink bugs into aeration chambers, defensive compounds were still obtained in large quantities on the first day of extraction (Table 1 and Fig. 2). Thus, these extracts were not used for bioassays or for fractionation. Extracts obtained from the second day onward contained low levels of defensive compounds (Fig. 2), and thus were used for analyses and bioassays.

The sesquiterpenes were seen in all extracts (*N*=150) collected from males, whereas these compounds were seen only in trace quantities in a small proportion (15 of 150) of female extracts (Table 1). β-Sesquiphellandrene was never seen in any of the 150 extracts from females that were analyzed. Furthermore, β-sesquiphellandrene was detected only in extracts from sexually mature males (>8 d old) (Fig. 2).

The absolute configuration of β-sesquiphellandrene was determined by two indirect routes because its enantiomers were not resolved on the Cyclodex B GC column. First, the insect-produced β-sesquiphellandrene was dehydrogenated with 3,4,5,6-tetrachloro-1,2-benzoquinone to produce the aromatic analog, α-curcumene, which was resolved on the chiral column, with the (7*S*)-enantiomer eluting first (McBrien et al. 2002). The α-curcumene derived from β-sesquiphellandrene produced by male *P. guildinii* proved to be the (7*R*)-enantiomer. However, because the dehydrogenation reaction gave a poor yield of α-curcumene, a second method was developed to confirm the absolute configuration of the insect-produced β-sesquiphellandrene. Thus, standards of stereoisomerically pure (7*S*)-β-sesquiphellandrene isolated from ginger oil, synthetic (7*R*)- and (7*S*)-sesquiphellandrenes, and the insect-produced β-sesquiphellandrene were hydrogenated, producing a ~2.5:1 mixture of diastereomers. On the Cyclodex B column, the more abundant, earlier-eluting

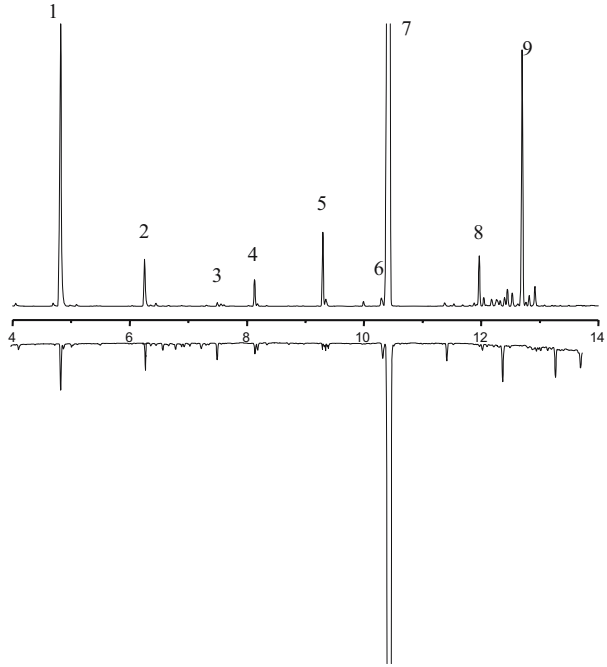


Fig. 2 Typical gas chromatograms of volatiles collected from male (top) and female (bottom, inverted) *P. guildinii* when the insects are not stressed: 1=(*E*)-2-hexenal, 2=ocimene isomer, 3=2-ethylhexanol, 4=undecane, 5=nonanal, 6=tridecene, 7=tridecane, 8=α-farnesene, 9=β-sesquiphellandrene

diastereomer was not resolved into its enantiomers. However, the minor diastereomer derived from each enantiomer was partially resolved [derivative from (*S*)-enantiomer, 133.13 min; from (*R*)-enantiomer, 134.01 min]. Coinjection of the reduced β-sesquiphellandrene collected from the insects with the reduced (7*S*)-β-sesquiphellandrene standard confirmed that the insect-produced material had the (7*R*)-configuration.

Analysis of 10 extracts obtained from groups of 20 males aerated for 24 hr determined that males released approximately 40 ng of sesquiphellandrene per bug per day. There was no clear cycle of production, with males producing similar amounts during morning, afternoon, and evening (Fig. 3, repeated measures ANOVA, $F_{2,8}$=0.21, P=0.81).

Bioassays with Live Insects Female bugs were attracted to odors of live males (χ^2_1 =10.76, N=41, P=0.001; Fig. 4) but not to odors of live females (χ^2_1 =0.13, N=32, P=0.72; Fig. 4). Males were not attracted to odors of females (χ^2_1 =0.28, N=14, P=0.59; Fig. 4) or of males. In the latter case, most of the males tested (24/30) did not leave the release chamber of the olfactometer, and so statistical analysis was not performed for the six individuals that made a choice (four to control, two to treatment; Fig. 4). When female and male *P. guildinii* were tested with the crude extract of males, female bugs were attracted to the extract of male odor (χ^2_1 =18.06, N=64, P<0.001; Fig. 5a), whereas males were not (Fig. 5a). These results indicated that males produced a sex-specific pheromone that attracted only females, and so all further bioassays were done only with females as test individuals, with the exception that males were also bioassayed to the synthetic standards to double-check that the compound was a sex pheromone rather than an aggregation pheromone.

Females' Responses to Crude Extract of Males at Different Times of the Day When females were tested during three different periods of the day (morning, afternoon, and evening), they responded to crude extracts of male-produced volatiles only in the evening. During the

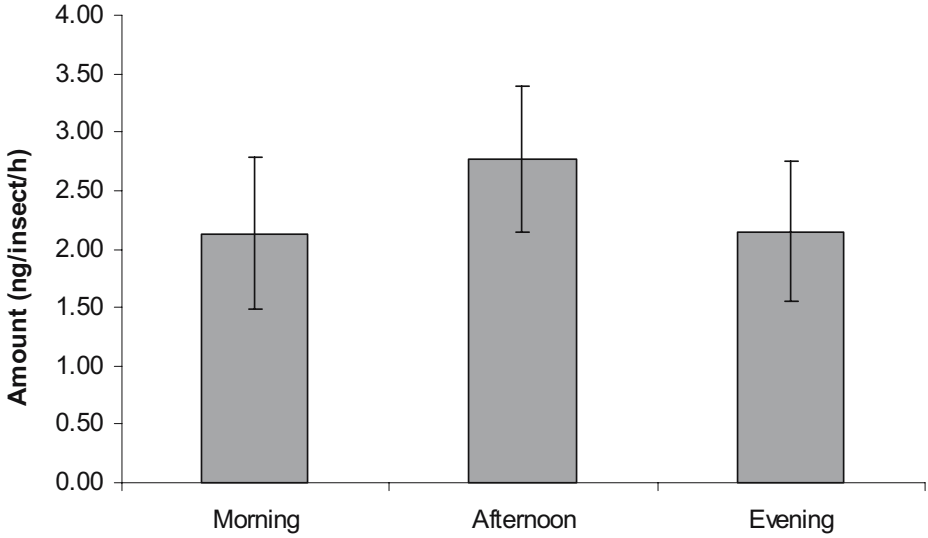


Fig. 3 Mean amount (ng insect^{-1} h^{-1}±SD) of pheromone released by male *P. guildinii* during different periods of the day. Collections lasted 5 hr for each period. The amounts collected during the three periods were not significantly different (repeated measures ANOVA, $F_{2,8}$=0.35, P=0.72)

evening, 70.3% of the females tested chose the treatment chamber (χ^2_1 =25.83, N=53, P<0.001; Fig. 5b), whereas for the morning only 4.3% (1/23) of females tested left the release chamber of the olfactometer (Fig. 5b). In the afternoon, the test insects were more active (69.5% moving out of the release chamber) but their responses were not oriented toward the treatment chamber

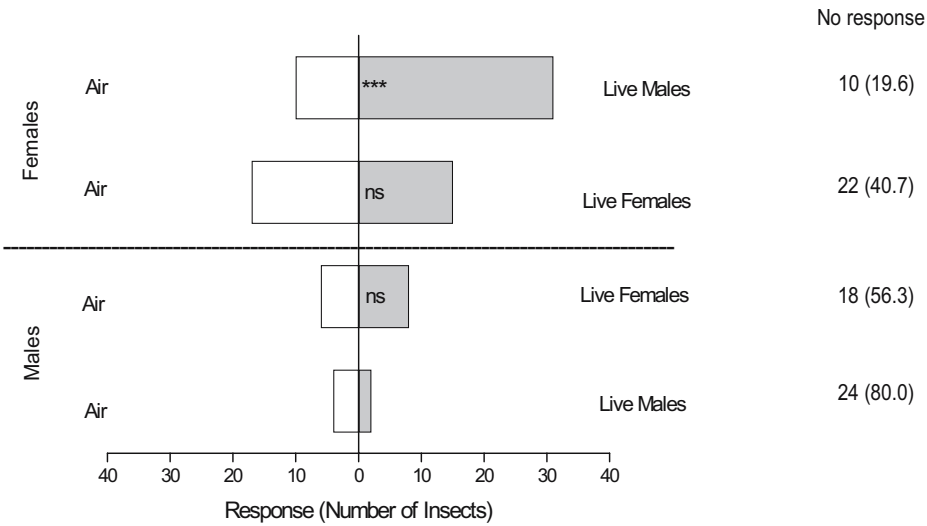


Fig. 4 Responses of male and female *P. guildinii* to odor of conspecifics of each sex vs. blank controls, in a two-choice olfactometer bioassay. Data were analyzed by chi-square tests (ns P>0.05, ***P<0.001). Data for males responding to odor of live males were not analyzed because of the low number of responders (6/30 insects tested). The numbers of nonresponders are to the right of the figure. Numbers in parentheses represent the percentage of nonresponders out of the total number of insects tested

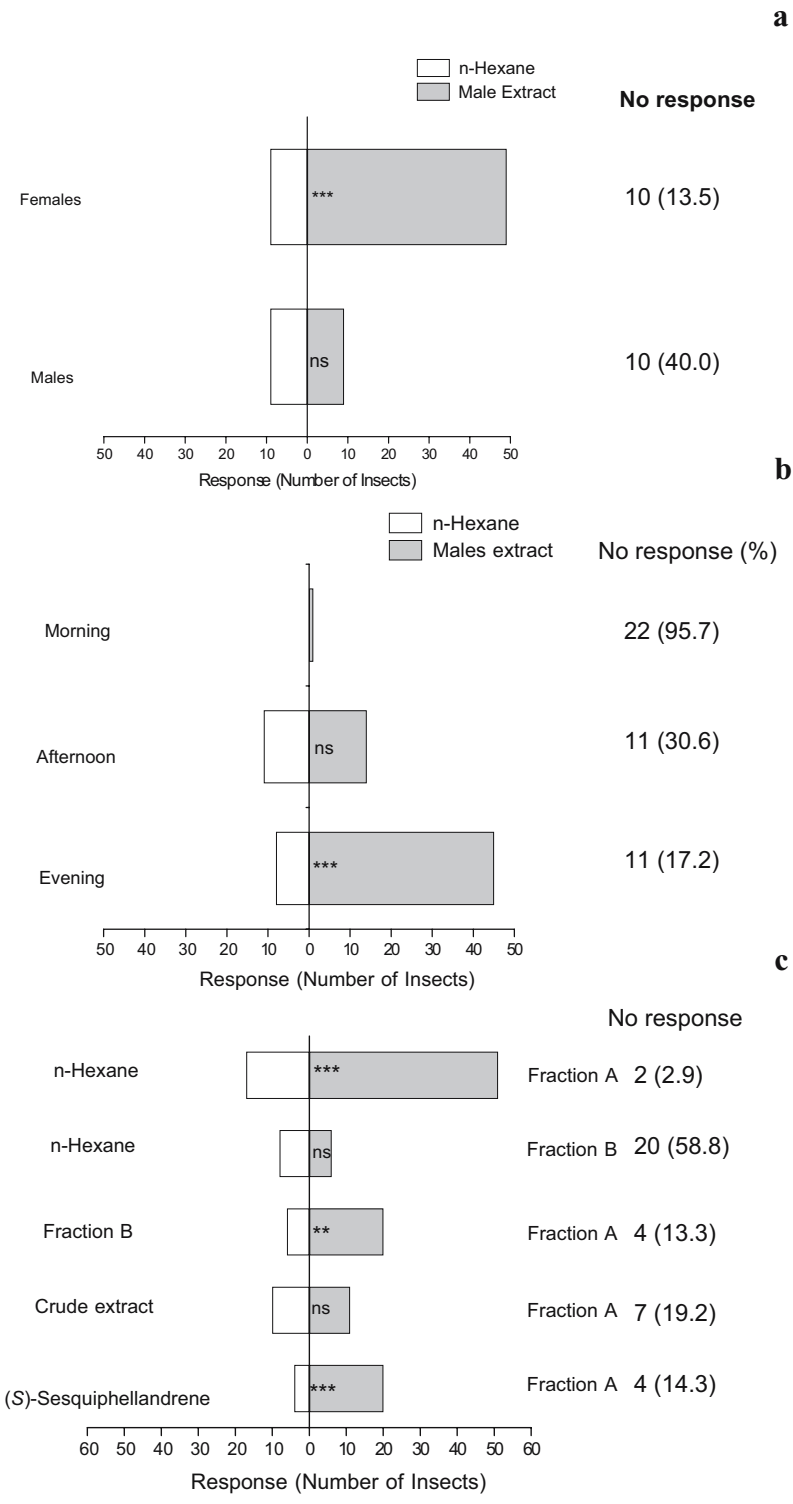
(χ^2_1 =0.36, N=25, P=0.55; Fig. 5B). Thus, during this period the females did not appear to be responsive to male pheromone. In light of these results, all further bioassays were performed from 17:00 to 22:00 hours.

Bioassays with Fractions of Insect Extracts and Synthetic Standards When the two fractions of crude extract were bioassayed against the hexane control, female *P. guildinii* responded to fraction A, which contained primarily (7*R*)-β-sesquiphellandrene (χ^2_1 =17.0, N=68, P<0.001; Fig. 5c). Females did not respond to fraction B, which contained primarily saturated hydrocarbons and several sesquiterpenes (χ^2_1=0.28, N=14, P=0.59; Fig. 5c). Females also preferred fraction A when it was evaluated directly vs. fraction B (χ^2_1 =7.54, N=26, P=0.006; Fig. 5c). There were no differences in the responses of female bugs to fraction A in bioassays against the crude extract (χ^2_1 =0.05, N=21, P=0.83; Fig. 5c), indicating that fraction A was responsible for the attractiveness of the crude extracts. Furthermore, when fraction A, containing (7*R*)-β-sesquiphellandrene, was tested against an equal amount of the pure (7*S*)-enantiomer isolated from ginger oil, fraction A was more attractive (χ^2_1 =10.67, N=24, P=0.001; Fig. 5c). In addition, when synthetic (7*R*)- and (7*S*)-β-sesquiphellandrenes were tested individually against hexane controls, the (7*R*)-β-sesquiphellandrene was attractive to females, whereas the (7*S*)-enantiomer was not (χ^2_1 = 4.83, N=35, P=0.03; Fig. 6a). In contrast, male bugs showed no response to either enantiomer of the synthetic sesquiphellandrene against hexane controls (Fig. 6a). When a 1:1 mixture of synthetic (7*R*)- and (7*S*)-β-sesquiphellandrenes was tested against each enantiomer individually, only the (7*R*)-β-sesquiphellandrene was as attractive as the 1:1 mixture (χ^2_1 = 0.33, N=48, P=0.56; Fig. 6b). However, when synthetic (7*R*)-β-sesquiphellandrene was tested vs. synthetic (7*S*)-β-sesquiphellandrene, females responded equally to both treatments (χ^2_1 =0.09, N=46, P=0.77; Fig. 6b). There was no difference in the response of female bugs to fraction A in bioassays vs. synthetic (7*R*)-β-sesquiphellandrene (Fig. 6b), indicating that fraction A, containing male-produced (7*R*)-β-sesquiphellandrene, was as attractive as synthetic (7*R*)-β-sesquiphellandrene to females. Furthermore, when fraction A was tested against the synthetic (7*S*)-enantiomer, fraction A was more attractive than the (7*S*)-enantiomer (χ^2_1 =10.67, N=24, P=0.001; Fig. 6b).

Discussion

Daily collections of volatiles (N=150) from groups of 20 *P. guildinii* males, starting 5 d after the final molt, and continuing for 15 consecutive days, showed that young males did not produce detectable levels of male-specific compounds. Male-specific compounds were first detected in extracts of males 8 d after the molt to adulthood. Although the pheromone was collected from male *P. guildinii* throughout the day, female bugs were only attracted to volatiles from males during the evening, suggesting that females are only responsive during this period. These results are analogous to previous reports of mating behavior of the stink bugs *Euschistus heros* and *Thyanta perditor* in which the strongest responses to pheromone occurred during late afternoon to early evening (Borges et al. 1998a,b; Moraes et al. 2005b).

Most of the putative defensive compounds identified in the headspace volatiles of *P. guildinii* adults consisted of compounds that have been reported previously from other neotropical stink bug species (Borges and Aldrich 1992; Moraes et al. 2005b; Borges et al. 2006). However, we found that, in addition to β-sesquiphellandrene, males consistently released several sesquiterpenes. In contrast, sesquiterpenes were only seen in trace amounts


a
n-Hexane
Male Extract
No response
Females
***
10 (13.5)
Males
ns
10 (40.0)
50 40 30 20 10 0 10 20 30 40 50
Response (Number of Insects)
b
n-Hexane
Males extract
No response (%)
Morning
22 (95.7)
Afternoon
ns
11 (30.6)
Evening
***
11 (17.2)
50 40 30 20 10 0 10 20 30 40 50
Response (Number of Insects)
c
No response
n-Hexane
***
Fraction A 2 (2.9)
n-Hexane
ns
Fraction B 20 (58.8)
Fraction B
**
Fraction A 4 (13.3)
Crude extract
ns
Fraction A 7 (19.2)
(S)-Sesquiphellandrene
***
Fraction A 4 (14.3)
60 50 40 30 20 10 0 10 20 30 40 50 60
Response (Number of Insects)

in a small percentage of extracts from females. The function of these sesquiterpenes, and why their production is strongly male-biased, is unknown. They did not appear to have a role in sexual attraction because (7*R*)-β-sesquiphellandrene alone was as attractive as crude extracts. They also do not appear to be artifacts from the green beans provided as food because they were not detected in extracts of volatiles from the green beans.

The first study of pheromones within the genus *Piezodorus* was carried out by Leal et al. (1998) with *P. hybneri*. The pheromone blend of *P. hybneri*, which attracted both sexes in laboratory bioassays, consisted of β-sesquiphellandrene (absolute configuration not determined), (*R*)-15-hexadecanolide, and methyl (*Z*)-8-hexadecenoate (ratio 10:4:1). The responses of females to subsets of the blend or individual compounds were not investigated (Leal et al. 1998). Thus, it remains unclear whether the full three-component blend is required to attract females, or whether the attraction observed in bioassays was actually because of only one or two components of the blend.

In contrast, the pheromone of congeneric *P. guildinii* appears to consist of a single component, (7*R*)-β-sesquiphellandrene, which attracts only females. Furthermore, examination of the percentage of nonresponders in bioassays (Figs. 4, 5, and 6b) showed that in all bioassays in which the natural compound (7*R*)-β-sesquiphellandrene was present (live males, airborne extracts, fraction A from airborne extracts), the percentages of females that did not respond were lower than those in bioassays in which the treatments did not include this compound (Figs. 4, 5, and 6b). It is also interesting to note that β-sesquiphellandrene produced by *P. guildinii* has the opposite configuration to the (7*S*)-β-sesquiphellandrene produced as a pheromone component by two other stink bug species, *Thyanta pallidovirens* and *Thyanta custator accerra* (McBrien et al. 2002).

Although the results from most of our bioassays indicated that the insect-produced (7*R*)-enantiomer of β-sesquiphellandrene was the active antipode, in one test similar numbers of bugs responded to both synthetic enantiomers (Fig. 6b). In addition, in some bioassays testing synthetic compounds, there were more nonresponding test bugs than in bioassays with the insect-produced material (Fig. 6). The cause of these anomalies is not clear. It may have been because of the fact that the synthetic compounds were both mixtures of epimers, rather then pure compounds, thereby distorting the olfactory signal. It was not because of large amounts of the (7*R*)-enantiomer in the synthetic (7*S*)-enantiomer because the latter compound was highly enantiomerically pure (>98% ee).

In a preliminary report, Borges et al. (1999) reported the identification of methyl 2,6,10-trimethyltridecanoate and methyl 2,6,10-trimethlyldodecanoate in extracts of field-collected *P. guildinii*. They (Borges et al. 1999) reported that these compounds appeared to be attractive to *P. guildinii*, based on catches in traps, that were deployed to catch *E. heros*. For *E. heros*, these two esters have been identified in volatiles collected from male bugs, and the biological activities of the esters verified in both laboratory bioassays and field tests (Borges and Aldrich 1994; Borges et al. 1998a,b; Costa et al. 2000; Zhang et al. 2003). In contrast, these compounds were not found in any of the samples analyzed in the current

Fig. 5 Response of *P. guildinii* adults in two-choice olfactometer bioassays. **a** Responses of males and females to odor extracts of males vs. solvent controls. **b** Responses of females to extracts of males vs. solvent controls during different periods of the day. Results from the morning period were not analyzed because only 1 of 23 insects responded. **c** Responses of females to crude extracts of males, fractions A [containing (7*R*)-β-sesquiphellandrene] and B (containing saturated hydrocarbons and several other sesquiterpenes) of crude extracts of males, and (7*S*)-β-sesquiphellandrene isolated from ginger oil vs. solvent controls. Data were analyzed with chi-square tests (ns $P>0.05$, ** $P<0.01$, *** $P<0.001$). The numbers of nonresponders are to the right of the figure. Numbers in parentheses represent the percentage of nonresponders out of the total number of insects tested

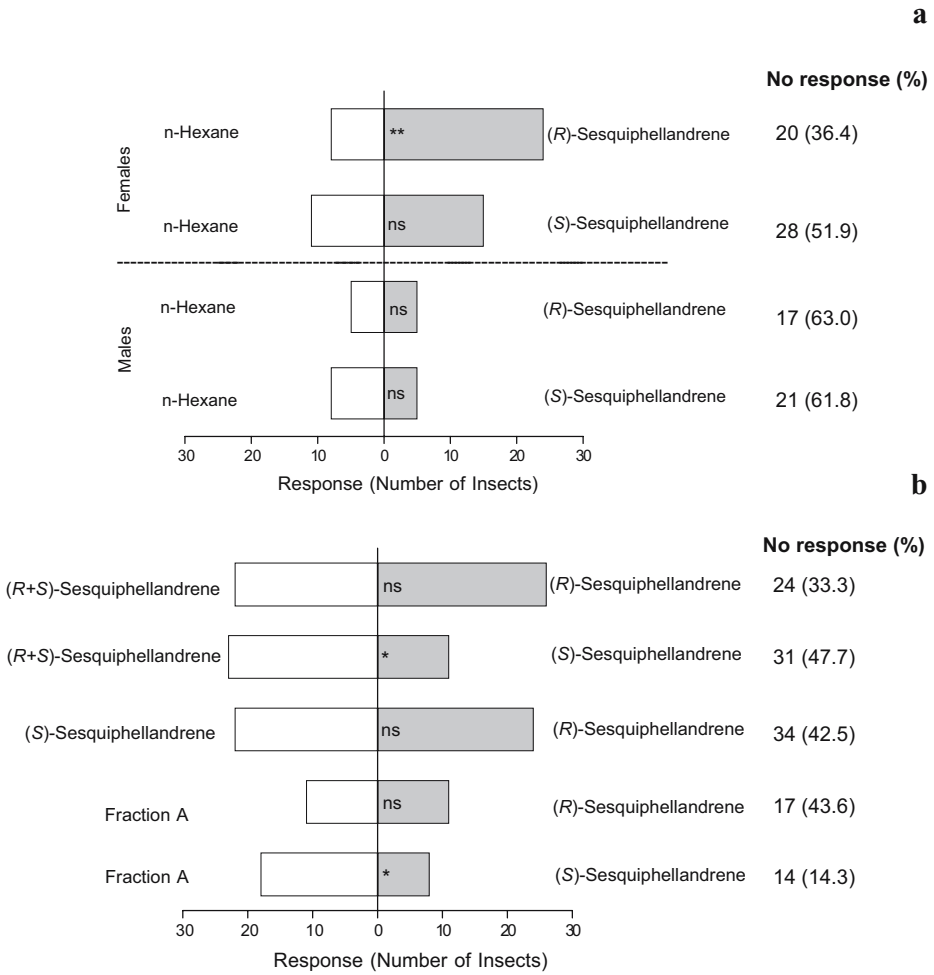


Fig. 6 Responses of *P. guildinii* in two-choice olfactometer bioassays. **a** Response of males and females to synthetic (7*R*)- or (7*S*)-β-sesquiphellandrene vs. solvent controls. **b** Response of females to synthetic (7*R*)- or (7*S*)-β-sesquiphellandrene vs. each other, vs. a 1:1 mixture of both compounds, and vs. fraction A [containing insect-produced (7*R*)-β-sesquiphellandrene]. Data were analyzed with chi-square tests (ns $P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). The numbers of nonresponders are to the right of the figure. Numbers in parentheses represent the percentage of nonresponders out of the total number of insects tested

study. It should be pointed out that the previous identifications were based only on GC retention time matches to the two esters, which on more detailed investigation by gas chromatography–mass spectrometry appear to be coincidental. The basis for the attraction of *P. guildinii* to the esters (Borges et al. 1999; Aldrich et al. 1993) is unclear. However, a similar phenomenon has been observed for other stink bug species in which bugs appear to be attracted to the pheromone components of other bug species. For example, the Asian species *Halyomorpha halys* (Stål) is attracted to ester pheromone components of *Plautia stali* (Scott) and of *Thyanta* spp., although the esters have not been found in *Halyomorpha* bugs (J. R. Aldrich, personal communication). In addition, Endo et al. (2007) reported cross attraction of *P. hybneri* to the aggregation pheromone of *Riptortus clavatus* (Thunberg).

The role of (7*R*)-β-sesquiphellandrene as a mediator of *P. guildinii* reproductive behavior under field conditions still remains to be determined. It is likely that the pheromone may act in concert with the substrate-borne vibrational signals that this insect uses for communication over shorter ranges (Moraes et al. 2005a). It may be possible to develop pheromone-based monitoring systems for this insect, particularly if vibrational signals could also be incorporated into the trap design. Such monitoring systems, when used in conjunction with strategies such as trap cropping, may provide an integrated pest management solution for control of this important pest.

Acknowledgements We thank Hélio Santos, Diva Tiburcio, and Camila Raquel for helping with field collection and laboratory rearing of the insects used in this study. This work received financial support from the Brazilian Council for Scientific and Technological Development (CNPq), Distrito Federal Research Foundation (FAPDF), and EMBRAPA. This research was also supported by the International Foundation for Science, Stockholm, Sweden, through a grant to Maria Carolina Blassioli Moraes.

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J Chem Ecol (2007) 33:1249–1256
DOI 10.1007/s10886-007-9304-3

A Male Sex Pheromone in a Scorpionfly

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Received: 1 December 2006 / Revised: 22 March 2007 / Accepted: 30 April 2007 /
Published online: 12 May 2007
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Abstract It has been postulated that males of a number of scorpionfly species produce sex pheromones. This is based on the observation that females often respond only to conspecific males when they evert their genital pouch, the proposed site of pheromone release. In this study, we prove that in *Panorpa germanica* (Mecoptera, Panorpidae), the eversion of a male's genital pouch is associated with the release of a volatile sex pheromone. In dual choice situations, females showed a high preference for 'calling' (males with everted genital pouch) over noncalling individuals. Volatiles emitted by males and females were collected and identified by coupled gas chromatography–mass spectrometry. Two aldehydes [(2*E*,6*Z*)-nona-2,6-dienal and (*E*)-non-2-enal] were characteristic of calling males but not of non-calling or immature males or females. Bioassays with synthetic compounds confirmed that the identified substances are attractive to females. To the best of our knowledge, this is the first identification of a sex pheromone in scorpionflies.

Keywords Sex attraction · (2*E*,6*Z*)-Nona-2,6-dienal · (*E*)-Non-2-enal · Male pheromone · Female mate choice · Scorpionflies · Mecoptera · *Panorpa germanica*

Introduction

In insects, sex attraction and pair formation is often mediated by the release of volatile sex pheromones by the female or, less commonly, the male (Wyatt 2003). Based on behavioral studies, the existence of male-derived sex attractants has long been suggested for Bittacidae (Bornemissza 1964, 1966a, b) and Panorpidae (Thornhill 1973, 1979, 1992) within the

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scorpionflies (Mecoptera). However, the chemicals involved in the sexual communication of scorpionflies have not been identified.

Felt (1895) and Grell (1942) suggested that chemical communication existed in *Panorpa* scorpionflies. Felt (1895) first speculated that a 'peculiar organ' formed by two processes at the dorsum of the third and the fourth segment of the male abdomen was the source of a volatile oil that attracts females. However, four decades passed before Grell (1942), in his detailed study on the morphology of the genital bulb of *P. communis*, described a layer of glandular epithelium in the outward septum of the male genital pouch and concluded this was the possible source of substances that sexually stimulate females during courtship. Thornhill (1979) found that females of nine different North American *Panorpa* species responded only olfactorily to conspecific males if the male's genital pouch were everted, confirming this structure to be linked to pheromone release.

Males of several Central European species extend their genital pouch to varying degrees during courtship, being most evident in *P. germanica* (Fig. 1), prominent in *P. alpina* and *P. cognata*, but less noticeable in *P. vulgaris* and *P. communis* (Sauer, personal observation). The 'calling' behavior of *P. germanica* is conspicuous as the male raises his abdomen and extrudes the genital pouch between the two separated ventral parameres of its genital segment (Fig. 1). At the same time, the male spreads and regularly flutters his wings—potentially helping to spread the emitted volatiles. These substances are detectable by humans, having a scent somewhat reminiscent of freshly cut cucumber. Male 'calling' and initiation of mating occurs only over a rather narrow time frame in the last 5 hr of the day and in the hour after sunset (Gerhards 1999). Females approaching a calling male will show interest by wing flapping and gentle nodding of the abdomen, but for mating to occur, the male must present the female a salivary secretion as a nuptial gift (Gerhards 1999). While males of *P. germanica* are polygynous, females usually mate only once or twice during their lifetime (Gerhards 1999). Consequently, male mating success (number of copulations obtained during life) is highly variable (Gerhards 1999), and we assume female choice to be a strong selective force in this system. Conceivably, female mate choice in this species could take place before copulation, based on the males' pheromone bouquets.

The aim of the present study was to investigate whether presentation of the eversible pouch by male *P. germanica* is correlated with the release of a volatile sex pheromone and to identify the involved chemicals.

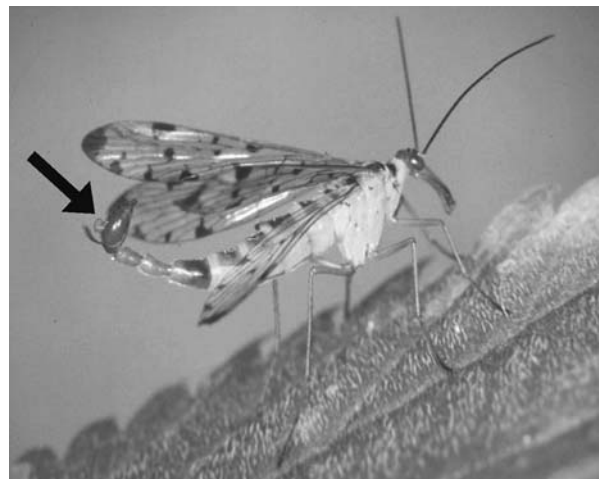


Fig. 1 A 'calling' *Panorpa germanica* male with everted genital pouch (indicated by the *arrow*)

Methods and Materials

Breeding and Keeping of P. germanica Adult *P. germanica* males and females collected in the field near Freiburg i. Br., southern Germany, were used to establish a colony under established rearing procedures (see Sauer 1970, 1977; Kock et al. 2006). The F1 imagoes were kept separately in plastic cylinders (diameter=3.5 cm, height=8 cm) containing moist tissue paper as a water supply under a 18:6 hr light/dark cycle at 18°C. All adult scorpionflies were fed with one segment of a last larval instar of mealworm (*Tenebrio molitor*) at the day of hatching and subsequently every fourth day. Under these conditions, *P. germanica* adults take about 8 d after emergence to reach sexual maturity. As *P. germanica* exhibits courtship display only at the end of the photophase and beginning of the scotophase, we reared some individuals under the same photoperiodic conditions but with lights off occurring 5 hr earlier so that we had both calling and noncalling males available during the normal mating window.

Volatile Sampling An apparatus designed for closed-loop stripping analysis (CLSA; Grob and Zürcher 1976) was used to prepare headspace extracts from (a) greater than 10-d-old mature calling (N=10) and (b) mature noncalling males (N=11), as well as (c) less than 6-d-old immature males (N=7) and (d) mature females (N=9). Individuals were placed in an Erlenmeyer flask (100 ml) equipped with a wash bottle insert that was connected to a mini-vacuum pump (Neolab, Heidelberg, Germany) by Teflon tubing. Volatile laden air (80 ml/min) was sucked for 1 hr through a CLSA adsorption tube (Gränicher & Quartero, Daumazan, France) containing a 1-mm charcoal layer (5 mg). The adsorbed substances were eluted with 25 µl dichloromethane containing 20 ng/µl of methyl nonanoate as an internal standard for approximate quantification of the collected volatiles. For males, it was recorded if the genital pouch was everted during volatile sampling.

Gas Chromatography–Mass Spectrometry Volatile extracts were analyzed by gas chromatography–mass spectrometry (GC-MS) on a Fisons 8060 GC (Fisons Instruments) equipped with a 30 m×0.32 mm inner diameter×0.25 µm film thickness DB-5 ms column (J&W Scientific, Folsom, CA) operated in splitless mode (injector temperature=240°C) and coupled to a Fisons MD800 quadrupole MS running in the electron impact (EI) mode at 70 eV. Helium was used as carrier gas at a head pressure of 5 kPa (flow rate, 1.0 ml/min). Initial oven temperature was 80°C, increased at 5°C/min to 280°C, and held for 30 min. Chemicals were identified by comparison of retention times and mass spectra with those of authentic reference compounds (Sigma-Aldrich, Steinheim, Germany). An approximate quantification of identified compounds was obtained by comparison of peak areas with the internal standard and between treatment values compared using a one-way analysis of variance (ANOVA) and subsequent least significant difference test (LSD).

Bioassays Bioassays on preferences for 'genuine' male odors, as well as for synthetic chemicals, were done by exposing females of *P. germanica* to different dual choice situations (see below) in Y-tube olfactometers constructed entirely from acrylic glass (each arm 50 cm in length and an inner diameter of 5 cm). To eliminate visual cues, targets were placed in opaque chambers at the end of two arms, and the female was then placed at the end of the third. A constant airflow of 50 l/hr was generated in both test and control arms. To eliminate possible positional bias, test and control arms were altered after every individual assay. Y-Tubes were cleaned regularly with 70% ethanol.

In experiment 1, females (N=68) were given the choice between a synthetic blend of (2*E*,6*Z*)-nona-2,6-dienal (17 mg) and (*E*)-non-2-enal (170 mg; Sigma-Aldrich) dissolved in

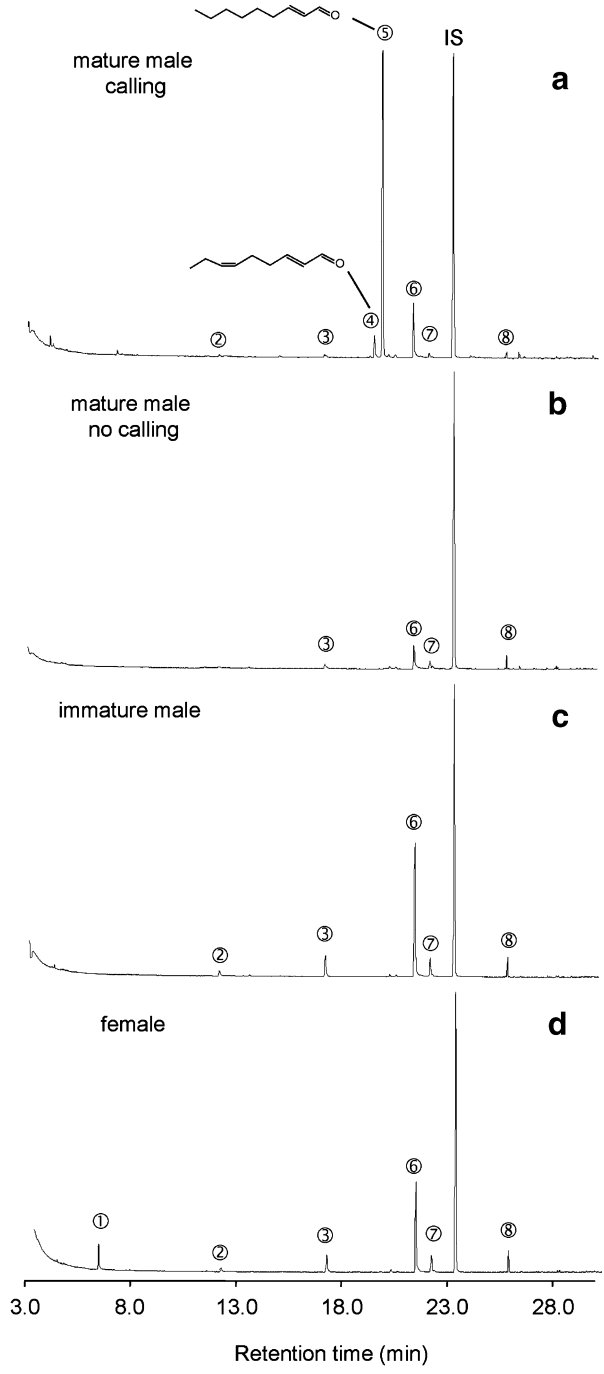


Fig. 2 Total ion current chromatograms obtained by GC-MS analysis of headspace extracts of **a** calling mature males, **b** non-calling mature males, **c** immature males, and **d** mature females. Numbered compounds were identified as butane-1,3-diol (*1*), decane (*2*), undecane (*3*), (2*E*,6*Z*)-nona-2,6-dienal (*4*), (*E*)-non-2-enal (*5*), 2-(2-butoxyethoxy)-ethanol (*6*), dodecane (*7*), tridecane (*8*); *IS* internal standard (20 ng methyl nonanoate). The remaining trace components are unknown

10 ml of polyethylene glycol (PEG) and the pure solvent as control. The selected ratio of the two aldehydes was based on the chemical analyses of headspace collections from calling males (see below). Five hundred microliters of the solutions were transferred into a commercially available membrane dispenser (Langlock® Do 120, Wilhelm Biological Plant Protection, Sachsenheim, Germany) that releases even highly volatile semiochemicals at constant emission rates (Ruther and Mayer 2005). The permeable membrane top cover, the volatile release site, was initially dampened with the PEG solution and then kept moist by gently and regularly shaking the flask. The estimated amounts of synthetic chemicals released from the dispensers was determined by sampling for 1 hr using the same procedure as described above for the volatile collections from insects (N=5).

In experiment 2, females (N=57) were given the choice between a calling (everted genital pouch) and a noncalling mature male (genital pouch not everted). In these trials, females were transferred into the Y-tube olfactometer when one male started calling and the other male exhibited no courtship display.

In both treatments, the behavior of each female was observed for up to 30 min, recording if the female moved into one of the two target sides of the Y-tube olfactometer. If a female showed courtship behavior (wing flapping) and, in addition, passed at least two thirds of the length of one of the target sides, the according target (calling male, noncalling male, synthetic aldehydes, control solution) was noted as the female's decision. If no such behavior occurred within 30 min, 'no decision' was recorded as the female's response to the dual choice situation.

Possible preferences of females that made a choice were analyzed by a binominal test.

Results

Chemical Analyses GC-MS analyses of headspace extracts from (a) mature calling males, (b) mature noncalling males, (c) immature males, and (d) mature females revealed two compounds characteristic of mature calling males (Fig. 2, Table 1) that were identified as (2*E*,6*Z*)-nona-2,6-dienal and (*E*)-non-2-enal (Fig. 3). Trace amounts of both compounds were detectable in volatile extracts from mature females, whereas noncalling mature males only emitted minute amounts of (*E*)-non-2-enal, and immature males released neither. Butane-1,3-diol, *n*-alkanes with chain lengths between 10 and 13 carbon units, and 2-(2-butoxyethoxy)-ethanol (probably a contamination from the insect container) were also

Table 1 Amounts of (2*E*,6*Z*)-nona-2,6-dienal and (*E*)-non-2-fenal collected from mature calling males, mature noncalling males, immature males, and mature females within 1 hr CLSA sampling

Gender	Condition	Number	mean amount±SE (ng/hr)	
			(2*E*,6*Z*)-nona-2,6-dienal[a]	(*E*)-non-2-enal[b]
Male	Mature, calling	10	21±6a	240±60a
Male	Mature, no calling	11	0.0±0.0b	1±1b
Male	Immature	7	0.0±0.0b	0.0±0.0b
Female	Mature	9	0.1±0.1b	4±3b

Different lowercase letters indicate significant differences within each column at P<0.001 (one-way ANOVA, LSD test).

[a] ANOVA (df=3): F=11.91, P<0.001.

[b] ANOVA (df=3): F=14.21, P<0.001.

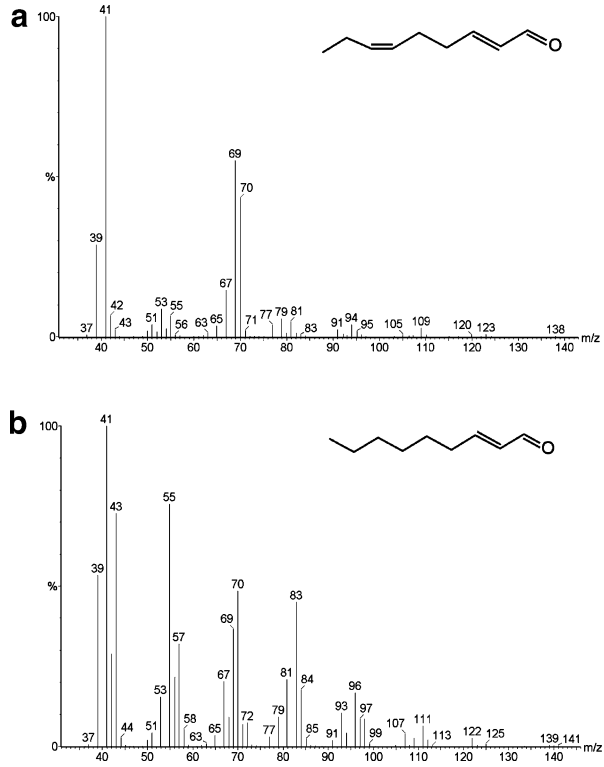


Fig. 3 Mass spectra (EI, 70 eV) of volatiles released by calling *P. germanica* males **a** (2*E*,6*Z*)-nona-2,6-dienal and **b** (*E*)-non-2-enal

detected, but none of these volatiles was specific for any group of insects. The two nine-carbon aldehydes were released by calling males at a ratio of approximately 1:10 in favor of (*E*)-non-2-enal. The membrane dispenser used for the bioassays released 570±60 ng/hr of (2*E*,6*Z*)-nona-2,6-dienal and 5,800±700 ng/hr of (*E*)-non-2-enal. Thus, the dispensing system released amounts equivalent to approx. 24 calling males at a ratio comparable to the natural male odor.

Bioassays Thirty-two out of 68 females (47%) made a choice in the Y-tube olfactometer that tested synthetic aldehydes vs. the solvent control, and 31 chose the arm with the synthetic chemicals ($P<0.001$, binomial test, Fig. 4). In experiment 2, 37 of 57 (65%) females tested made a choice between the calling vs. noncalling male, with 36 preferring the arm containing the calling male ($P<0.001$, binominal test, Fig. 4). By comparing the

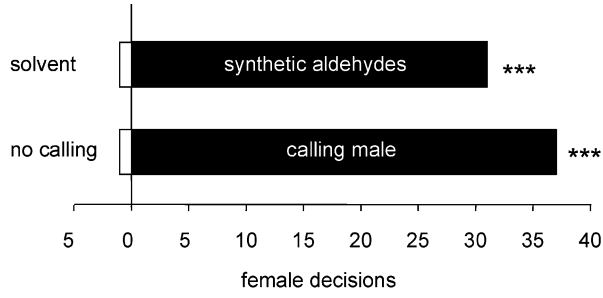


Fig. 4 Female decisions in Y-tube olfactometer preference tests. Females were given the choice between synthetic (2*E*,6*Z*)-nona-2,6-dienal and (*E*)-non-2-enal (1:10) dissolved in PEG and the pure solvent (*top*) or a calling and a noncalling mature male (*bottom*)

two treatments, we found that the number of nonresponders was significantly higher in the experiment that used the synthetic aldehydes (G test, G=3.88, P<0.05).

Discussion

Our results demonstrate that the calling behavior (eversion of the genital pouch) of mature *P. germanica* males is associated with the release of (2*E*,6*Z*)-nona-2,6-dienal and (*E*)-non-2-enal at a ratio of approximately 1:10. Calling males, as well as a mixture of the synthetic aldehydes, are highly attractive to unmated females in the Y-tube olfactometer, so we conclude that the two substances are male sex attractants for this species. To the best of our knowledge, this is the first identification of a sex pheromone in a scorpionfly.

Interestingly, the two aldehydes are major constituents of the odor bouquet of freshly cut cucumber, and they contribute significantly to the characteristic aroma of this vegetable (e.g., Forss et al. 1962; Kemp et al. 1974; Whitfield and Last 1991). Preliminary tests show freshly cut cucumber is attractive to *P. germanica* females in the Y-tube olfactometer. When cucumber fruits are damaged, these aldehydes are formed by enzymatic degradation of unsaturated fatty acids (Galliard and Matthew 1976), but it remains to be determined if the synthesis of pheromone compounds in insects follows the same pathway.

The responsiveness of females in the olfactometer tests was relatively low to both calling males and synthetic lures. In the latter case, the release rates from the dispensers were much higher than from calling males, so the low responses observed may be related to the concentration of the signal. In the case of virgin males, it could be related to mate choice. Unlike many other *Panorpa* species, such as *P. vulgaris* (Sauer et al. 1998, 1999), *P. communis* (Aumann 2000), and *P. cognata* (Engqvist and Sauer 2001, 2003) that are polyandrous, *P. germanica* females are generally monandrous (Kock and Sauer, unpublished data). In the case of the polyandrous *P. vulgaris*, there is no precopulatory mate choice, although females adjust the duration of copulation based on male quality, thereby receiving greater numbers of sperm from high-quality males (Sauer et al. 1997, 1998, 1999). In contrast, monandrous *P. germanica* females may exhibit precopulatory mate choice based on their ability to detect and evaluate the pheromone composition of potential mates. Thus, they will respond only to better quality males. This idea is supported by the fact that *P. germanica* females often refuse to mate if offered only a single male (Kock, personal observation). Additional research is required to determine if there is intermale variability in the ratio of the pheromone blend and whether females can detect and use such differences. We will also investigate whether less volatile substances, which act only at close range, play a role in precopulatory mate choice by this species.

Acknowledgements Financial support was provided by the Deutsche Forschungsgemeinschaft (DFG; SA 259/7—1 to 4). J. Ruther was supported by a Heisenberg fellowship (grant RU 717/7-1) of the DFG.

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J Chem Ecol (2007) 33:1257–1271
DOI 10.1007/s10886-007-9291-4

Perception of Conspecific Female Pheromone Stimulates Female Calling in an Arctiid Moth, *Utetheisa ornatrix*

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Received: 28 September 2006 / Revised: 23 February 2007 / Accepted: 13 March 2007 /
Published online: 14 April 2007
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Abstract Perception of the female sex pheromone in *Utetheisa ornatrix* (Lepidoptera: Arctiidae) is responsible for induction and adjustment of calling by females and the collective phenomenon termed "female pheromonal chorusing". We found five olfactory-active compounds in the *U. ornatrix* female gland. When females were exposed to the entire pheromone or to two of its (synthetically prepared) components, (*Z*,*Z*,*Z*)-3,6,9-eicosatriene and (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene, they were more likely to call during a given night, begin calling earlier, and briefly increase signal frequency with which they extrude their abdomen, an observable indication of calling in this species. Some females even initiated calling during photophase when exposed to the pheromone components. In general, female *U. ornatrix* are more sensitive to the complete blend of pheromone than to its individual compounds. We also tested the hypotheses: 1) that abdominal extrusion *per se* increases the rate of pheromone release; and 2) that greater abdominal pumping rhythm increases pheromone release rate. Contrary to our expectations: 1) females did not respond more strongly to a pulsed pheromone stimulus than to the constant release of pheromone at

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the same average release rate; and 2) we did not find a relationship between the frequency of abdominal pumping and pheromone release rate. Possible explanations for these unexpected findings are discussed.

Keywords Arctiid moth · Chemical communication · Olfactory response · Sex pheromone · Signal perception

Introduction

In lepidopteran communication, females are typically regarded as non-interactive advertisers before courtship and pair formation. They are believed to signal their receptivity and location to males via long-distance pheromones. Potential responses to the sex pheromones of neighboring females are generally overlooked. However, some neurophysiological and behavioral evidence suggests that the latter may not be accurate for some moth species, and that communication by females may be more interactive and complex than previously considered. Researchers have found electrophysiological responses of female antennal neurons to conspecific female sex pheromone components in several lepidopteran families: Arctiidae (*Panaxia quadripunctaria*, Schneider et al. 1998; *Utetheisa ornatrix*, Grant and O'Connell 2000), Noctuidae (*Heliothis virescens* and *H. subflexa*, Groot et al. 2005; Hillier et al. 2006; *Spodoptera littoralis*, Ljungberg et al. 1993), and Sphingidae (*Manduca sexta*, Kalinová et al. 2001). Conspecific females and/or their sex pheromone are also known to induce calling in females in Tortricidae (*Choristoneura fumiferana*, Palaniswamy and Seabrook 1978, 1985; *Cydia fagiglandana* and *C. splendana*, Den Otter et al. 1996), and to attract (Tortricidae; *C. fagiglandana* and *C. splendana*; Den Otter et al. 1996) or repel other females (Noctuidae; *H. armigera* and *H. zea*, Saad and Scott 1981).

At a basic level, pair formation in the arctiid moth *U. ornatrix* conforms to the general feature of female-emitted pheromones eliciting upwind flight in conspecific males. Female *U. ornatrix* begin calling at dusk and normally continue for several hours. As in various arctiid moths, calling behavior is highly apparent and entails pumping of the abdomen, i.e., rhythmically extruding the terminal segments that bear the pheromone glands, at a rate of 1.5–3.0 sec^{-1} (Conner et al. 1980, 1985). Three active components of the *U. ornatrix* female pheromone have been identified: (*Z*,*Z*,*Z*)-1,3,6,9-heneicosatetraene (1,*Z*3,*Z*6,*Z*9-21Hy), (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene (*Z*3,*Z*6,*Z*9-21Hy), and (*Z*,*Z*)-6,9-heneicosadiene (*Z*6,*Z*9-21Hy) (Conner et al. 1980; Jain et al. 1983). Importantly, electroantennogram (EAG) responses to one of the components, *Z*3,*Z*6,*Z*9-21Hy, occur in females (Grant and O'Connell 2000) and in males (Conner et al. 1980; Jain et al. 1983).

A recent study reported that female *U. ornatrix* within olfactory range of neighboring females synchronize the onset of their daily calling periods and exhibit a higher abdominal pumping rate than isolated individuals (Lim and Greenfield 2007). Grouped females were also more likely to call on a given night, and to call without interruption and for a longer duration than females that were olfactorally isolated from neighbors (Lim and Greenfield 2007). These data show that females modify the timing and intensity of calling in the presence of neighboring females, a phenomenon termed "female pheromonal chorusing" (Lim and Greenfield 2007). Evidence that females respond specifically to conspecific sex pheromone is circumstantial, although as pointed out by Grant and O'Connell (2000), "gender-specific body odors" may conceivably play a role in generating the interactive behavior. In the present study, we examine the specific responses in calling of *U. ornatrix*

females to conspecific female sex pheromone, and its separate components, to understand the mechanisms and evolution of this communal sexual display.

In a previous study, Lim and Greenfield (2007) reported that calling females increased abdominal extrusion rates in response to the presence of calling neighbors. We propose that the extrusion rate indicates the "vigor" with which calling is performed, and is related to energetic expenditure during calling, and pheromone release rate. The latter is based on the hypothesis that rhythmic abdominal pumping serves to increase peak pheromone release rate, thereby maximizing potential attraction of males (Schal and Cardé 1985; Dusenbery 1989). Alternative adaptive hypotheses for abdominal pumping include the creation of an additional axis of the pheromone signal, temporal modulation, along which species specificity may be encoded, and the reduction of sensory adaptation in the male receiver (Conner et al. 1980). Therefore, we examined two additional questions concerning the potential response of female *U. ornatrix* to the conspecific female pheromone: Do females exhibit a heightened response to a pulsed pheromone signal, and does the abdomen extrusion rate relate to the pheromone release rate?

Methods and Materials

Study Population A laboratory population of *U. ornatrix* was bred from moths collected in Highlands County, Florida, USA. Larvae were maintained on a standard pinto-bean diet (Shorey and Hale 1965), supplemented with 20% (*w*/*w*) of seeds of *Crotalaria mucronata*, the host plant of *U. ornatrix* at the collection site (Dussourd et al. 1991), in an environmental chamber at 26°C and 14L:10D photoperiod. Moths from the sixth through ninth generations, from the time of collection from the field, were used for the experiments. Inbreeding was minimized by establishing 10 maternal lines from the original field collection (ca. 300 caterpillars) and out-crossing them. To ensure standard physiological and behavioral states among experimental animals, we only used virgin individuals and tested them once on the second or third day after eclosion, i.e., adult individuals aged 24–48 hr.

Pheromone Gland Extraction Pheromone gland extracts were prepared from individual *U. ornatrix* female moths and analyzed at different times of the diel period, by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). For preparation of the extracts, the terminal 3 or 4 abdominal segments (bearing the pheromone gland) of a female were dissected with microscissors, and placed individually in a vial containing 100 µl of hexane and a mixture of 100 ng each of (*Z*)-5-tridecene and (*Z*)-7-tetradecene as internal standards. Dissection of the pheromone glands was performed 2 hr before (N= 8 females) and 2 hr after (N=40 females) the onset of scotophase. These times were chosen because females rarely call during photophase and mostly attain peak abdominal extrusion rate ca. 2 hr after the onset of scotophase (Lim and Greenfield 2007). Of the 40 individuals tested during scotophase, 26 were calling at the time of gland dissection. The glands were removed from the vial after 30 min and the extracts stored at −20°C until GC analysis and behavioral bioassay.

Gas Chromatography and Electroantennographic Detection A Hewlett-Packard (HP) 5890 Series II gas chromatograph, equipped with either a DB-5 or DB-225 column (30 m× 0.25 mm ID, J & W Scientific, Folsom, CA, USA) and a 50:50 effluent split, was used for gas chromatography-electroantennographic detection (GC-EAD) analysis. Helium was the carrier gas, with a flow rate of ca. 30 ml min^{-1} for both FID and EAD. Before GC analysis,

the gland extracts were concentrated to 10–20 μl, by using a gentle stream of nitrogen, and 1 μl injected into the GC in splitless mode. Initial oven temperature was 60°C, held for 1 min, then increased to 250°C for DB-5 and 200°C for DB-225 at 10°C min^{-1}. The outlet for the EAD had a purified and humidified air stream flowing over the antennal preparation at 600 ml/min.

Gas chromatography-mass spectrometry (GC-MS) analyses of gland extracts were performed with an HP 5890 Series II GC interfaced to an HP 5972 Mass Selective Detector. The GC-MS was equipped with a DB-5 column (identical to that used in the GC-EAD system), which was temperature-programmed as for the GC-EAD analyses. Electron ionization mass spectra were recorded from 30 to 550 amu at 70 eV. Chemicals were identified by comparison of retention indices and mass spectra with those of authentic standards. Two synthetic compounds, (*Z*,*Z*,*Z*)-3,6,9-eicosatriene (*Z*3,*Z*6,*Z*9-20Hy) and *Z*3,*Z*6,*Z*9-21Hy, were provided by Russell Jurenka (Iowa State University, Ames, IA, USA). Isomeric and chemical purity of these two compounds were >99.5%, as determined by GC.

Whole-body preparations were used for EAD experiments. A virgin male or female moth was immobilized on a plasticine block with small U-shaped copper wires, and its antennae were further restrained with thinner copper wires. A glass microelectrode (tip diameter≈ 1 mm, 0.86 mm I.D., A-M Systems Inc., Washington, USA) filled with 0.1 M KCl solution was introduced through the cuticle of the distal part of the antenna and served as a recording electrode; another glass microelectrode penetrated a compound eye to serve as a reference electrode. Silver–silver chloride junctions were used to maintain electrical contact between the electrodes and amplifier. The EAD signal was first amplified with a high-input impedance (>10^{12} Ω) headstage preamplifier (Syntech®, Hilversum, The Netherlands), and further processed with a PC-based signal processing system (Syntech®, Hilversum, The Netherlands).

Bioassay of Calling To test the influences of female pheromone on calling behavior, we presented females with filter paper discs (2 cm diam., Whatman® no. 1) impregnated with *U. ornatrix* female pheromone gland extract or synthetic compounds, *Z*3,*Z*6,*Z*9-20Hy and *Z*3,*Z*6,*Z*9-21Hy. *Z*3,*Z*6,*Z*9-20 Hy was a newly identified olfactory-active compound for both sexes of *U. ornatrix* (see "Results"); therefore, we tested it to evaluate its function in female–female pheromonal interaction. We could not test synthetic 1,*Z*3,*Z*6,*Z*9-21Hy because of its chemical instability (Huang et al. 1983), but we tested the responses of females to synthetic *Z*3,*Z*6,*Z*9-21Hy.

One equivalent of gland extract (IE), the mean amount of pheromone extracted from a naturally calling female during scotophase, was used as a standard unit throughout the experiments. Preliminary GC-EAD and behavioral assays showed that *U. ornatrix* females are sensitive to hexane (personal observation). Therefore, each filter paper disc was dried in an airflow for 30 sec before presentation in our behavioral bioassays.

We monitored the behavior of each of the test females during 15-sec observations made every 5–15 min throughout a 60- to 120-min period during early scotophase at 25±1°C. During these observations, we noted: (1) whether or not the female was calling and, if yes, (2) the number of abdominal extrusions per 15 sec. To avoid observer bias, a double-blinded observer was randomly assigned for each test. From these data, we determined the following three indices of each individual female's calling: (1) the time each individual was first observed calling, with scotophase onset designated as 0 min; (2) the mean signal frequency, measured in abdominal extrusions per minute, of each individual showing calling behavior during at least one observation; (3) the total time that an individual spent

calling, inferred from the number of observations when she was observed calling (Lim and Greenfield 2007). For all data, we listed the time of a reported event as the midpoint of the 5- to 15-min cycle of observations during which it was seen.

Call Induction During Photophase We evaluated whether pheromone could induce females to call by testing its effect during mid-photophase, when *U. ornatrix* ordinarily do not call. Single females were placed in individual glass cells ($d \times l$ = 1 cm×5 cm), connected by tubing to a chemical source at one end and to an exhaust at the other (flow rate <10 cm/sec). Test individuals were placed in cells at least 6 hr before the onset of scotophase, and their behavior observed every 15 min over the following 5 hr at 25°C. We initiated observations 6 hr before scotophase onset to avoid the possibility that the circadian rhythms of some females might trigger calling near the end of photophase (Silvegren et al. 2005).

No behavioral responses were observed from females to 1 IE during photophase. Therefore, we repeated the trials using higher quantities of gland extracts (5 IE, N=50; 10 IE, N=19; 15 IE, N=15) and synthetic *Z*3,*Z*6,*Z*9-21Hy (100 ng, N=16; 200 ng, N=18; 400 ng, N=10; Conner et al. 1980). A *post hoc* analysis indicated that 100 ng of *Z*3,*Z*6,*Z*9-21Hy was approximately equivalent to the mean quantity found in 1 IE (see "Results "). As a control, we tested 48 individuals with filter paper discs containing hexane only.

Call Stimulation During Scotophase Using the apparatus described earlier, we tested the influence of both pheromone gland extracts and synthetic pheromone components on calling behavior of female *U. ornatrix* during scotophase. Ten (N=17) or 20 ng (N=15) of *Z*3,*Z*6,*Z*9-20Hy (N=32 sets of six females), equivalent to the mean quantity in 1 IE and 2 IE, respectively, 50 (N=24 sets) or 100 ng (N=10 sets) of *Z*3,*Z*6,*Z*9-21Hy (N=34 sets), equivalent to the mean quantity in 0.5 IE and 1 IE, respectively, or 1 IE (N=11 sets), 2 IE (N=13 sets), 4 IE (N=15 sets), 6 IE (N=15 sets), and 10 IE (N=20 sets) of gland extract (N=74 sets), were tested. We also tested additional sets (N=54) of six individuals with the control stimulus. We made 15-sec observations for each individual every 5 min for 1 hr after odor presentation. For a given set of six females tested together in the apparatus, we recorded: (1) time of calling first observed for each individual, (2) proportion of individuals calling, and (3) mean signal frequency, expressed as the average of the maximum signal frequencies of individual moths showing calling behavior.

Calling Acceleration During Scotophase We tested whether signal frequency of calling females increased after exposure to pheromone. As described in the previous section, we monitored signal frequencies of 42 individual females and recorded the frequency during and after exposure to 0.05 IE of gland extract. We used the low quantity of 0.05 IE to test only the immediate behavioral response to the pheromone. Because signal frequency usually reaches its peak between 50 and 90 min after the onset of calling (Lim and Greenfield 2007), we presented the pheromone 20 min after the onset of calling to avoid coinciding with the natural increase in signal frequency. We observed and recorded the signal frequency of each individual during three consecutive 5-min observation periods: 5 min before and 5 min after pheromone presentation, and 5 min thereafter. During the observation, signal frequencies of individual moths were counted each and every minute. Filter paper discs containing only hexane were tested as control (N=17).

Calling Stimulation by Pheromone Pulsing Because calling in *U. ornatrix* females entails a rhythmic pulsing of pheromone release, we tested whether females responded to temporal

modulation of pheromone concentration *per se*. Specifically, we examined whether calling females were more likely to accelerate their signal frequency to higher values when exposed to pulsed pheromone than to a steady stream of pheromone, the release rate of which was identical to the mean value of the pulsed one. To pulse the pheromone stream, we punctured a small hole in the tube upstream from the chemical stimulus (filter paper disc) and tapped a finger on the hole at a rate of 3 sec^{-1}, the maximum signal frequency observed in *U. ornatrix* females (Conner et al. 1980, 1985; Lim and Greenfield 2007). We first presented the odor 15 min after scotophase onset and then began tapping 10 min later and continued for 5 min. The 10-min delay between odor presentation and the onset of tapping allowed us to measure the signal frequency after exposure to both continuous and pulsed stimuli. We used 0.05 IE as the stimulus in 18 trials of six individuals each. We confirmed that the finger tapping generated a puff at the location of the test individuals by simulating it with a visible marker (incense smoke). A control volatile (filter paper disc with hexane only) was tested in 15 parallel trials of six individuals each. We recorded the signal frequency of each individual during three 5-min periods as follows: 5 min before the tapping during tapping, and during the 5 min after tapping. During each period, we measured the signal frequency of each individual five times, each measurement being the number of abdominal extrusions in 15 sec. To test whether the pheromone puff *per se* influenced female calling, we also compared the signal frequencies of the females exposed to the pulsed pheromone with the group tested with a steady stream of pheromone in the previous test.

Signal Frequency and Pheromone Release Rate We tested the hypothesis that the signal frequency is positively related to pheromone release rate during calling and that grouped females, who generally call at elevated signal frequencies during the beginning of the scotophase, are releasing pheromone at higher rates. We established two different airborne pheromone collection apparatus, one for "solitary" and one for "grouped" females. In each apparatus, we placed females singly in individual cells, observed their calling behavior and signal frequency, and collected and quantified pheromone released. Solitary females were held in individual glass cells such that they were not exposed to the pheromone of neighbors, whereas grouped females were held in individual screen cells that exposed them to other females' pheromone.

We conducted 12 solitary female trials, in which 10 individual females were held singly within 15-ml glass tubes that were all connected to a Tenax odor trap. To prevent the solitary females from perceiving their neighbors' pheromone accumulating in the collection apparatus, and to enhance collection efficiency, air was exhausted from the apparatus through the odor trapper at a flow rate of 30 cm/sec. We also conducted 12 grouped female trials, in which eight individual females were held singly within screen cells placed in a 12-cm diam Petri dish connected to a Tenax odor trapper. We used different numbers of test individuals in the solitary (10 females for each collection) and grouped trials (eight females for each collection) so that the total number of calling individuals in a trial would be equivalent; typically, in previous tests, we had observed that 50% and 70% of solitary and grouped individuals, respectively, called (Lim and Greenfield 2007). Each of the screen cages holding the grouped females were separated by at least 0.5 cm to prevent direct physical contact between the moths. As in the solitary female trials, air was exhausted from the apparatus through the odor trap at a rate of 30 cm sec^{-1}. The Tenax odor trap consisted of a glass Pasteur pipette ($l \times d = 5 \times 0.5$ cm) packed with 300 mg of pre-cleaned Tenax (35/60 mesh, Alltech®, Deerfield, IL, USA) held in place by glass wool plugs.

In both solitary and grouped female trials, volatile chemicals were collected for 5 hr after onset of scotophase. While collecting pheromone, we observed calling behavior by each

female every 10 min. We estimated pheromone release rates of individual females by dividing the total amount of pheromone collected by the total signaling time in a trial (equal to the sum of total signaling durations of each of the 10 solitary or eight grouped females in the trial). Three grouped females and 10 solitary females that either died before or oviposited while in the apparatus for the 5-hr trial were not included in the analyses. After collection, 100 ng each of (*Z*)-5-tridecene and (*Z*)-7-tetradecene were added to the Tenax trap as internal standards, and the Tenax rinsed four times with 400 μl of hexane. The solution was concentrated under a gentle stream of nitrogen to a final volume <50 μl and analyzed by GC. Unless otherwise noted, variation in all analyses is reported as mean± standard deviation.

Results

Pheromone Components Gas chromatography-electroantennographic detection (GC-EAD) and GC-MS analyses confirmed the presence of the three previously identified pheromone compounds, *Z*6,*Z*9-21Hy (Retention time=8.88 min), *Z*3,*Z*6,*Z*9-21Hy (Rt=9.25 min), and 1,*Z*3,*Z*6,*Z*9-21Hy (Rt=9.43 min) in our *U. ornatrix* population. Both males and females showed strong antennal responses to all three compounds. Besides these compounds, the analyses revealed two additional GC-EAD-active compounds. One was identified as *Z*3,*Z*6, *Z*9-20Hy by comparison of retention time (8.6 min) and mass spectrum with the synthetic compound (Lim, unpublished data). This compound elicited EAD responses from both male and female antennae. The other compound, which had a shorter retention time (8.52 min), elicited responses from male, but not female, antennae. Its identity was not determined in the present study. We also found two distinct profiles of gland extracts from females. In one profile, *Z*3,*Z*6,*Z*9-21Hy was the major component, whereas in the other 1, *Z*3,*Z*6,*Z*9-21Hy was the major component; there was no detectable amount of *Z*3,*Z*6,*Z*9-21Hy in females of the latter profile type. For this study, we did not discriminate these two profiles in our bioassays.

During photophase, only 1,*Z*3,*Z*6,*Z*9-21Hy was found in gland extracts (N=8, 44.76± 54.09 ng/individual). In scotophase, 1,*Z*3,*Z*6,*Z*9-21Hy remained as the major component in general (Fig. 1), although the quantities of all the components varied greatly. The total quantity of the five compounds in gland extracts (354.20±286.47 ng/individual, N=26 calling individuals) prepared during scotophase was approximately eight times greater than that in photophase females (Mann–Whitney U test, W=52.0, P<0.001). There was a general difference in pheromone quantity between calling and non-calling females during scotophase (Fig. 1). The mean quantity of *Z*3,*Z*6,*Z*9-21Hy in calling females (98.72±170.11 ng/individual) was significantly greater than that in non-calling females (1.95±3.14 ng/ individual, Mann–Whitney U test, W=607.0, P=0.03), as was the mean quantity of the unidentified compound (12.41±12.16 ng/individual in calling, vs. 0.98±1.18 ng per individual in non-calling, females; Mann–Whitney U test, W=109.0, P=0.04). The quantity of 1,*Z*3,*Z*6,*Z*9-21Hy in gland extracts did not differ between calling (239.03±288.20 ng/ individual; N=26) and non-calling females (N=14, 80.71±122.49 g/individual, Mann–Whitney U test, W=587.5, P=0.13), although it was the major component. Similarly, the mean quantities of *Z*3,*Z*6,*Z*9-20Hy and *Z*6,*Z*9-21Hy in calling females (11.79±22.91 and 1.50±2.17 ng/individual, respectively) were not significantly different than those in non-calling ones (3.85±7.95 and 2.16±1.69 ng/individual, Mann–Whitney U test, W=588.5, and 75.0, P=0.09 and 0.37, respectively).

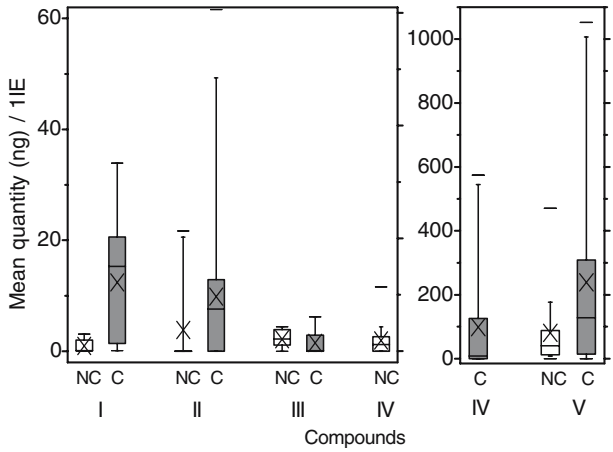


Fig. 1 Mean quantity (ng) of each pheromone component extracted from gland extract of individual females that were not calling (NC: open boxes) or calling (C: solid boxes) 2 hr after onset of scotophase. (I) unknown, (II) *Z*3,*Z*6,*Z*9-20Hy, (III) *Z*6,*Z*9-21Hy, (IV) *Z*3,*Z*6,*Z*9-21Hy, and (V) 1,*Z*3,*Z*6,*Z*9-21Hy. Box plots show means (X), medians (horizontal line), 25–75% ranges (rectangle), 5–95% ranges (inner horizontal bar), and minimum/maximum values (outer horizontal bar) of quantities

Call Induction During Photophase Both gland extract and synthetic *Z*3,*Z*6,*Z*9-21Hy induced calling from solitary females during photophase. No females tested began calling in response to the control discs or discs containing 5 IE, but 6 of 19 females responded to 10 IE, a significantly higher proportion than responding to the control (Fisher's exact test, $P<0.001$). Only 1 of 15 females responded to 15 IE (not different from the control, Fisher's exact test, $P=0.24$). Two of three quantities of *Z*3,*Z*6,*Z*9-21Hy elicited a calling response from significantly more test females compared with responses to the control disc: 5 of 16 to 100 ng (Fisher's exact test, $P<0.001$), 4 of 18 to 200 ng (Fisher's exact test, $P=0.004$), and 1 of 10 females to 400 ng (Fisher's exact test, $P=0.19$). Calling induced during photophase was generally brief, lasting only 3.02±2.78 min on average. The latency of call induction during photophase was generally shorter in response to gland extract (30.44±25.24 min; $N=9$) than to *Z*3,*Z*6,*Z*9-21Hy (92.5±70.67 min; $N=10$; Mann–Whitney *U* test, $W=124.0$, $P=0.05$). Mean signal frequencies of calling stimulated by *Z*3,*Z*6,*Z*9-21Hy (75.46±21.42 extrusions · min^{-1}, $N=9$) were lower than those stimulated by gland extract (138.89±29.33, $N=3$, Mann–Whitney *U* test, $W=45.0$, $P=0.02$).

Call Stimulation During Scotophase The bioassays indicated that both gland extract and individual pheromone components elicited significantly different calling responses during scotophase compared with the control. For each treatment, there was no significant difference in calling pattern in response to different concentrations (Kruskal–Wallis tests for gland extract and Mann–Whitney *U* tests for individual compounds, *ns*); therefore, calling responses were pooled. Females presented with gland extract or *Z*3,*Z*6,*Z*9-20Hy (1) were more likely to call (Kruskal–Wallis test, $H=20.80$, $df=3$, $P<0.001$; Dunn's pairwise comparison, $P<0.01$; but this effect was not found in response to *Z*3,*Z*6,*Z*9-21Hy, Dunn's pair-wise comparison, $P>0.05$; Fig. 2a); (2) started calling sooner (Kruskal–Wallis test, $H=19.92$, $df=3$, $P<0.001$; Dunn's pairwise comparison, $P<0.01$ and $P<0.05$, respectively; but this effect was not found in response to *Z*3,*Z*6,*Z*9-21Hy, Dunn's pairwise comparison, $P>0.05$; Fig. 2b); and (3) called with a higher signal frequency only in response to *Z*3,*Z*6,*Z*9-21Hy (Kruskal–Wallis test, $H=22.76$, $df=3$, $P<0.001$; Dunn's pairwise comparison, $P<0.01$; Fig. 2c), compared to females presented with the control stimulus.

Call Acceleration During Scotophase Exposure to 0.05 IE of gland extract elicited a brief increase (37%) in signal frequency from 106.43±46.29 to 145.36±31.91 extrusions min^{-1}

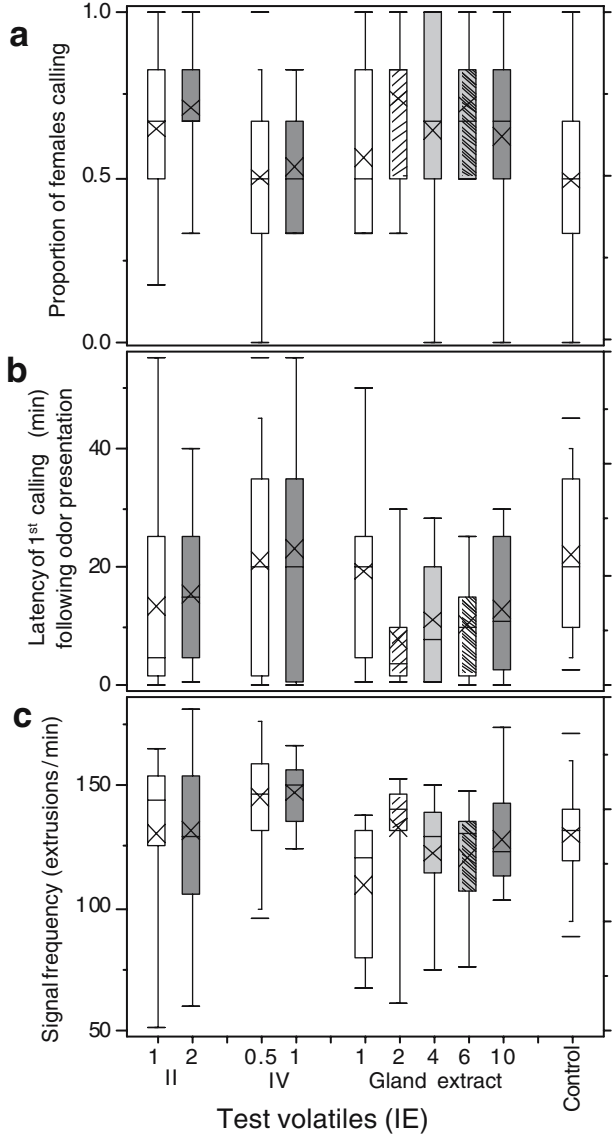


Fig. 2 Behavioral responses and temporal aspects of calling in individual females influenced by three different test volatiles and control. (II) *Z*3,*Z*6,*Z*9-20Hy and (IV) *Z*3,*Z*6,*Z*9-21Hy; 10 and 20 ng of II were equivalent to the quantity of the compound found in one and two female equivalents of gland extract, respectively; 50 ng and 100 ng of IV to 0.5 and 1 equivalents, respectively. (a) Proportion of calling individuals in a trial. (b) Latency (min) of first calling. (c) Signal frequency of calling individuals in each trial. Box plots show means (X), medians (horizontal line), 25–75% ranges (rectangle), 5–95% ranges (inner horizontal bar), and minimum/maximum values (outer horizontal bar) of values

during the 5-min presentation of the chemical stimulus. This increase was short-lived and females returned to values equivalent to pre-stimulus frequencies (119.40±47.26 extrusions · min^{-1}) shortly after removal of the stimulus (Repeated Measures ANOVA based on normally distributed data, N=42, F=25.24, df=2, 41, P<0.001; Fig. 3a). Control females tested in parallel did not show any evidence of change (signal frequencies=114.06±44.15, 119.88±45.15, and 118.18±40.40 extrusions · min^{-1}; N=17; Friedman's test, χ^2=1.20, df=2, P=0.55; Fig. 3a, open boxes).

Call Stimulation by Pheromone Pulsing Females increased their signal frequency from 117.75±30.04 to 147.54±25.06 extrusions · min^{-1} during the 5-min period when a

chemical stimulus (0.05 IE) was pulsed, and then returned to 122.81±28.70 after the cessation of pulsing (Friedman's test, N=67, χ^2=95.74, df=2, P<0.001). There was no change when clean air was pulsed (parallel values 108.55±27.55, 113.28±29.87, and 111.31±28.06 extrusions · min^{-1}; Friedman's test, N=58, χ^2=3.41 df=2, P=0.18).

When we compared peak signal frequencies of females exposed to, pulsed chemical stimulus, continuous chemical stimulus, pulsed clean air, and continuous clean air, we found that groups stimulated by either pulsed or continuous pheromone attained higher signal frequencies than groups stimulated by pulsed clean air (Kruskal–Wallis test, H= 39.83, df=3, P<0.001; Dunn's pairwise comparison, P<0.01; Fig. 4). However, we found no significant difference between signal frequencies of groups stimulated either by pulsed pheromone and continuous pheromone (Dunn's pairwise comparison, P>0.05) or by continuous pheromone and continuous clean air (Dunn's pair-wise comparison, P>0.05). Thus, the increase noted above and shown in Fig. 3b probably did not result from pulsed airflow *per se*, but rather was a short-term response to the onset of the chemical stimulus.

Signal Frequency and Pheromone Release Rate Our 5-hr collections showed that grouped and solitary females released comparable amounts of pheromone. *Z*3,*Z*6,*Z*9-20Hy was the

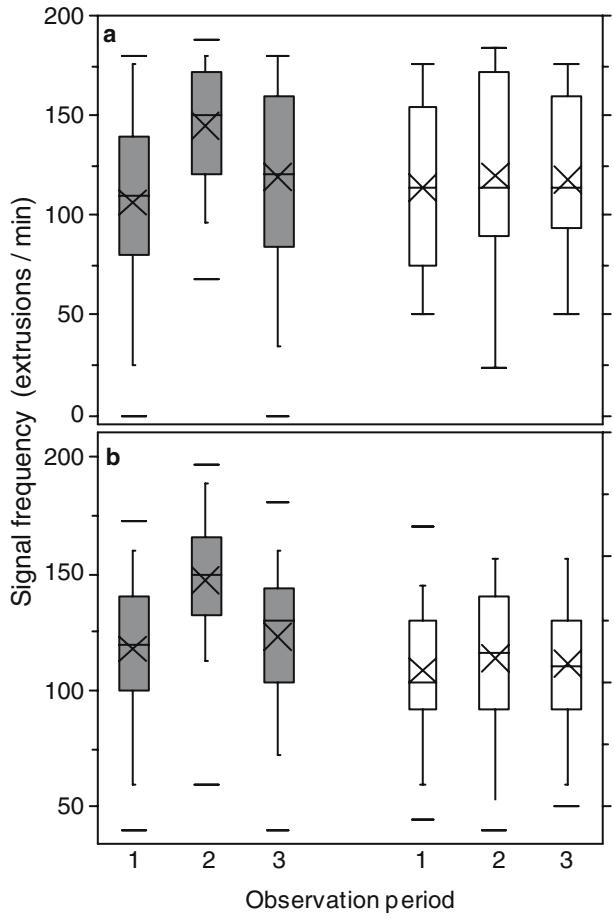


Fig. 3 (a) Mean signal frequency exhibited by females in response to gland extract (solid boxes) or control stimulus (open boxes). Observation periods: (1) 5-min before the odor presentation, (2) 5-min period after odor presentation, and (3) 5-min period after period 2. Data are taken from 42 test females and 17 control females. (b) Mean signal frequency exhibited by females in response to pulsed pheromone stimulus (solid boxes) or control stimulus (open boxes). Observation periods: (1) 5-min period with steady air stream containing pheromone before pulsing, (2) 5-min period of pulsing, and (3) 5-min period with steady air stream after period 2, respectively. Data are taken from 67 test females and 58 control females. Box plots show means (X), medians (horizontal line), 25–75% ranges (rectangle), 5–95% ranges (inner horizontal bar), and minimum/maximum values (outer horizontal bar) of frequencies

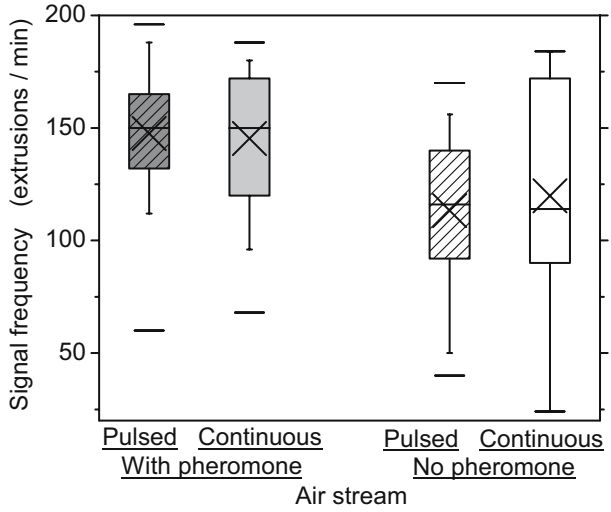


Fig. 4 Mean signal frequency exhibited by females in response to pulsed or continuous air with or without pheromone stimulus. Box plots show means (X), medians (horizontal line), 25–75% ranges (rectangle), 5–95% ranges (inner horizontal bar), and minimum/maximum values (outer horizontal bar) of frequencies

most abundant component released (7.90±7.87 ng/individual for solitary and 7.91±5.27 ng/individual for grouped females; sign test, $n+=7$, $n-=5$, ties=0, $P=0.39$). The amounts of *Z*3,*Z*6,*Z*9-21Hy (0.45±0.93 ng/individual for solitary and 0.08±0.27 ng/individual for grouped females; sign test, $n+=4$, $n-=1$, ties=7, $P=0.19$) and 1,*Z*3,*Z*6,*Z*9-21Hy (3.87±2.50 ng/individual for solitary and 4.22±4.26 ng/individual for grouped females; sign test, $n+=8$, $n-=4$, ties=0, $P=0.19$) also did not differ between solitary and grouped females. The mean pheromone release rates of grouped and solitary females were 0.98±0.92 and 1.03±0.71 ng · individual^{-1} · hr^{-1}, respectively (sign test, $n+=7$, $n-=5$, ties=0, $P=0.39$; Fig. 5).

Most grouped females in our test called at some time during the pheromone collection periods (mean proportion=0.85±0.14, $N=12$ trials), whereas a lower proportion of solitary females did (0.61±0.19, $N=12$ trials; sign test, $n+=10$, $n-=2$, ties=0, $P=0.02$). However, grouped and solitary females spent comparable amounts of time calling (129.90±27.47 min vs. 136.40±29.83 min, respectively; sign test, $n+=6$, $n-=6$, ties=0, $P=0.61$), and also

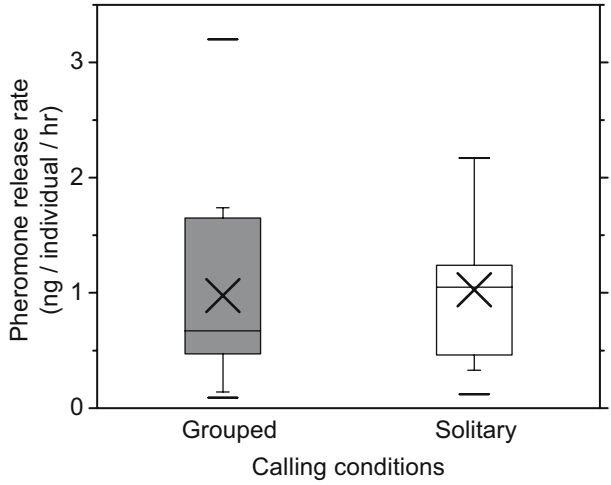


Fig. 5 Mean pheromone release rate (ng individual^{-1} hr^{-1}) of grouped and solitary calling females. Pheromone release rate is the total five compounds. Data are from 78 grouped females (solid box) and 66 solitary females (open box) that called during the collection period. Box plots show means (X), medians (horizontal line), 25–75% ranges (rectangle), 5–95% ranges (inner horizontal bar), and minimum/maximum values (outer horizontal bar) of quantities

exhibited comparable mean signal frequencies (117.48±12.75 extrusions · min^{-1} vs. 116.03± 16.66, respectively; sign test, $n+=7$, $n-=5$, ties=0, $P=0.39$) as well as peak signal frequencies (162.45±5.28 extrusions · min^{-1} vs. 156.91±12.97, respectively; sign test, $n+=6$, $n-=6$, ties=0, $P=0.61$).

Discussion

The chemical analyses and behavioral assays indicate that perception of the female advertisement pheromone is directly responsible for induction and modification of calling by *U. ornatrix* females. When *U. ornatrix* females perceive conspecific female pheromone, they (1) are more likely to call during that night, (2) begin calling earlier, and (3) accelerate their signal frequency to a higher rate during at least a brief interval. Some females even initiate calling during photophase, when such activity normally does not occur, in response to the pheromone or its individual components if presented in high enough concentration. In a previous study (Lim and Greenfield 2007), it was noted that females also increase the total amount of time spent calling during a night in response to the presence of neighboring females. However, the inability to present chemical stimuli in a controlled manner over a prolonged interval prevented us from evaluating the direct effect of sex pheromone on this aspect of calling in *U. ornatrix*.

Entire Pheromone Bouquet vs. Individual Components In general, we found that *U. ornatrix* females are as, or more, sensitive to the entire pheromone blend than to its individual components. During scotophase, both gland extract and *Z*3,*Z*6,*Z*9-20Hy elicited calling from more females and more quickly than did *Z*3,*Z*6,*Z*9-21Hy; the gland extract also elicited calling more quickly during photophase than did *Z*3,*Z*6,*Z*9-21Hy. However, during scotophase, *Z*3,*Z*6,*Z*9-21Hy increased signal frequency in calling females more than either the gland extract or *Z*3,*Z*6,*Z*9-20Hy. The results with *Z*3,*Z*6,*Z*9-20Hy should be viewed with caution, though, as the influence of this compound on orientation of males has yet to be tested either in the laboratory or the field; therefore, its role as a component of the female sex pheromone is not established (see Wyatt 2003).

In many moth species, the male is more sensitive to the entire pheromone bouquet than to its individual components (Cardé and Haynes 2004). In these cases, the sensitivity of males to the specific "blend ratio" of the components may reflect the importance of the ratio in species recognition and in the enhancement of "signal-to-noise ratio" for eliciting a behavioral response from the males. In other species, however, one (major) component may play a more important role throughout the full course of sexual communication and orientation than other (minor) components. Here, males may have an elevated neural sensitivity to the major component (e.g., Baker et al. 2004), and the minor components of the pheromone may function only during limited and specific parts of the pair-forming sequence (e.g., Vickers 2002).

Although many environmental and developmental factors including prior exposure to the female pheromone (Stelinski et al. 2005), prior exposure to host plant volatiles (Ochieng et al. 2002), rearing temperature (Linn et al. 1988), ambient temperature during sexual activity (Ono 1993), age (Greiner et al. 2002), hormonal status (Anton and Gadenne 1999), and circadian rhythm (Silvegren et al. 2005) may affect the sensitivity of pheromone processing in males, males of most moth species generally respond rapidly and selectively to minute changes in blend ratio and quantities of female pheromone components (Cardé

and Haynes 2004). This processing is necessary for locating a signaling female before other males do (Greenfield 2002). Female moths, on the other hand, may not be subject to similar selection pressures. Although females may perceive the conspecific female sex pheromone, it is not clear that they would be selected to discern the composition with the same degree of precision and speed as males. That is, females need only detect the presence of conspecific female pheromone within their vicinity and respond appropriately (Lim and Greenfield 2007). This may explain the comparable effects of the gland extract and some of the individual components in our study.

Influence of Pulsed Chemical Stimuli Contrary to our initial expectation, females did not respond more strongly to the pulsed pheromone stimulus than to the constant release of pheromone at the same average rate. This finding indicates that females may not respond to peak-to-peak changes in pheromone concentration but rather to time-averaged levels. However, the influence of pheromone pulsing on enhancing male responses has yet to be tested.

Signal Frequency and Pheromone Release Rates Along with a previous study (Lim and Greenfield 2007), our present study indicates increases in female signal frequency, the abdominal extrusion rate, in response to neighboring females and to pheromone. Earlier, we had proposed that this increased signal frequency yields a higher pheromone release rate. Contrary to this prediction, we found no difference between the pheromone release rates of solitary and grouped females. This lack of difference may reflect several factors. First, the low amounts of pheromone released, <1 ng $\cdot$ individual$^{-1} \cdot$ hr^{-1} (see "Results"), may have prevented us from resolving differences in release rates should they have occurred. Second, the 5-hr collection period (necessary to collect detectable amounts of pheromone; Jia et al. 2001; Lim and Greenfield 2007) may have masked any transitory changes in pheromone release rates caused by the abdominal pumping. Third, total calling times by solitary and grouped females may have also confounded our attempt to examine the relationship between signal frequency and pheromone release rate. Thus, at this time our data neither support nor unequivocally refute the hypothesis that the elevated signal frequency displayed by grouped females causes an increased pheromone release rate and a more intense signal to neighboring female signalers.

Acknowledgments We thank M. Deyrup of the Archbold Biological Station, Highlands County, Florida, for helping us collect *U. ornatrix* and its host plants. We also thank M. -Y. Choi, W. E. Conner, T. Eisner, V. Iyengar, and C. W. LaMunyon for providing critical information on *U. ornatrix* biology and pheromone analyses during various stages of the project and R. Jurenka for providing us with synthetic pheromone components. J. Kelly and two anonymous reviewers offered valuable critiques of earlier versions of the manuscript. K. M. Nus and J. A. Campbell of the Division of Biological Sciences at the University of Kansas took care of the host plants and provided test facilities. A. M. Swatek served as an invaluable laboratory assistant. The Hungerford Fund and the Entomology Graduate Student Summer Scholarship at the University of Kansas, Department of Ecology & Evolutionary Biology provided financial support.

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Introduction

Methyl eugenol (ME) is present as a component of leaf and floral essential oils from many plant species (Tan 2000; Tan et al. 2002, 2006). The strong affinity and voracious feeding behavior of some male tephritids exposed to ME has been a puzzling mystery since 1915 (Howlett 1915), and while the biological basis of this sex-specific attraction has not been fully understood, it has helped to eradicate the oriental fruit fly, *Bactrocera dorsalis*, in the islands of western Pacific by the "male annihilation technique" (Steiner and Lee 1955; Steiner et al. 1970; Koyama et al. 1984). Recent evidence has supported the role of ME as a pheromonal precursor in the mating ecology of *B. dorsalis* (Nishida et al. 1988; Shelly and Dewire 1994; Tan and Nishida 1996, 1998; Hee and Tan 1998; Shelly 2000a, 2001a). The fact that ME metabolites also act interspecifically as allomones against vertebrate predators (Tan and Nishida 1998; Wee and Tan 2001) has sparked the involvement of fruit fly attractants in a tritrophic level in the plant–fruit fly–predator relationship, apart from intraspecific (pheromone) and plant–fruit fly relationships (synomones) (Tan and Nishida 2000, 2005; Tan et al. 2002, 2006). Hence, ME, as a potent male attractant, has evolutionary and ecological significance in the life history of fruit fly.

B. dorsalis males produce (*E*)-coniferyl alcohol (CF) and 2-allyl-4,5-dimethoxyphenol and are sexually more competitive and successful in mating than ME-deprived males 1 d after ME consumption (Tan and Nishida 1996, 1998). Males of *Bactrocera carambolae* Drew and Hancock (a sibling species of *B. dorsalis*) are also attracted to ME, but sensitivity and attraction are significantly lower than that of *B. dorsalis* (Wee and Tan 2000; Wee et al. 2002). Furthermore, *B. carambolae* after ME consumption produced only CF along with its endogenous pheromonal components 6-oxo-1-nonanol (major component), *N*-(3-methylbutyl) acetamide, 1,6-nonanediol and ethyl 3-methyl-2-hydroxybutanoate (Tan and Nishida 1996; Wee and Tan 2005a). *B. carambolae* consumed a significantly lower quantity than *B. dorsalis* (189 vs. 735 μg) when given unlimited access to ME (Wee et al. 2002). We wondered, therefore, whether ME-fed *B. carambolae* males could gain a level of significant sexual advantage similar to that of its sibling species, *B. dorsalis*.

Here, we investigate the role of ME in sexual communication of *B. carambolae* by examining the volatile emissions from ME-fed males before and after courtship and by establishing the relationship between ME-feeding and male sexual advancement in courtship compared to ME-deprived males. Results were compared to those previously obtained for the sympatric and sibling species, *B. dorsalis* (=*Bactrocera papayae*).

Methods and Materials

Insects Laboratory strains of *B. carambolae* were pure-breeding lines for more than 30 generations. Larvae were cultured on a semi-artificial diet containing star fruit, yeast, protein, wheat bran, sugar, sweet potatoes, and water (Wee and Tan 2000).Flies were segregated by sex within 5 d after emergence. Virgin males and females were maintained separately at 30±2°C and relative humidity of 73±5% with a 12 L:12 D photoperiod. Adult food mixture, comprised of protein, yeast, sugar, and sodium benzoate, and water, was provided. Sexually mature 25- to 35-d-old (Wee and Tan 2000) virgin males and females were used.

Chemicals Methyl eugenol (1,2-dimethoxy-4-[2-propenyl]benzene; >99.8% purity) was obtained from Agrisense-BCS Ltd. (UK). (*E*)-Coniferyl alcohol (CF) (> 96% *trans*) and *N*-

(3-methylbutyl)acetamide (>95% purity) were previously synthesized (Nishida et al. 1990). 6-Oxo-1-nonanol was synthesized by reacting propyl magnesium iodide with a large excess of ε-caprolactone (Aldrich Chemical Co. Inc.) in ether, followed by chromatographic purification with a silica gel column and elution with 40% methyl acetate in hexane (>95% purity). Compounds were diluted to appropriate concentrations for chemical identification and internal standards in gas chromatographic (GC) analyses.

Methyl Eugenol Consumption by Males To differentiate between ME-fed and ME-deprived males in a field cage mating competition experiment, 15-d-old male fruit flies were divided into two cohorts (individuals of one cohort were marked with green or white nontoxic paint on the thorax by using a fine-headed pin) and maintained separately until required for the experiment. Color assignment was random for the first replicate and reversed for each subsequent replicate to eliminate any possible effect of color preferences by females, even though color was unlikely to affect mate choice.

To prepare ME-fed flies, groups of ten specific color-marked male flies were allowed to feed on 0.1 μl ME dispensed on a small piece of filter paper (3×3 cm^2; Whatman® No.1), placed in a disposable Petri dish, for 10 min during their peak response to ME (10:00–11:00 hours) (Wee and Tan 2000). Treated males used were either at 1, 2, or 3 d posttreatment (DPT) of ME, depending on experimental design.

Collection of Male Volatile Emissions B. carambolae is a dusk-mating species (Wee and Tan 2000). Therefore, volatiles emitted by live males were collected from (1) 16:00–18:00 hours (before sunset), (2) 18:00–20:00 hours (dusk), and (3) 20:00–22:00 hours (after sunset) by using a simple aeration setup.

Analytical grade compressed air was passed through a dryer (20×3 cm diameter) containing silica gel followed by a Tenax filter, to adsorb excessive moisture and possible organic impurities, before reaching a 3-l airtight glass chamber containing 100–150 virgin ME-fed males. Airflow was maintained at 40–60 ml/min. At the outlet end, the emission was trapped by an adsorbent trap, Tenax TA (5 mg, 50–80 mesh), held between plugs of silanized glass wool in a Pasteur pipette. The Tenax trap was purged with a continuous flow of helium gas (20–40 ml/min) and baked overnight at 270°C before use. The trap was replaced with a newly activated one every 2 hr, and then, eluted with 10-ml redistilled ethanol. The eluate was concentrated to ca 100 μl with a rotary evaporator under partial pressure. One microliter aliquots were subjected to gas chromatographic (GC) and GC-mass spectrometric (GC-MS) analyses, respectively.

Chemical Analyses GC analysis was conducted with a Shimadzu GC-14A gas chromatograph, equipped with a fused silica column (25 m×0.25 mm i.d.×0.33 μm) coated with nonpolar, cross-linked bonded dimethylpolysiloxane (HP Ultra-1). The carrier gas was helium, and the oven temperature was held at 80°C for 1 min then programmed at 10°C/min to 240°C. Injection mode was splitless, and detection was by a flame ionization detector (FID). Data were captured with a Shimadzu C-R6A integrator. Chemical identification was performed by comparing the retention time of authentic standards and co-elution of internal standards on GC.

GC-MS analysis was performed on an HP 5989B mass spectrometer (electron impact at 70 eV) connected to a fused silica column (30 m×0.25 mm i.d.×0.33 μm) coated with nonpolar, cross-linked 5% phenyl-methylpolysiloxane (HP-5MS) under the same conditions as above.

Chemical identity of rectal volatiles was confirmed by comparing the retention time and mass fragment pattern with those of synthetic samples or reported data on GC-MS: *N*-

(3-methylbutyl)acetamide (I): MS, *m/z* (%): M^+ 129 (15), 114 (13), 86 (28), 73 (85), 72 (68), 70 (13), 60 (33), 55 (21), 44 (81), 43 (100); ethyl benzoate (II): MS, *m/z* (%): M^+ 150 (28), 122 (29), 105 (100), 77 (47), 51 (20), 44 (14); benzamide (III): MS, *m/z* (%): M^+121 (76), 105 (100), 77 (95), 76 (12), 51 (44), 44 (30); 6-oxo-1-nonanol (IV): MS, *m/z* (%): M^+ 158 (6), 140 (7), 115 (23), 97 (48), 86 (58), 79 (15), 73 (19), 71 (100), 69 (87), 58 (65), 55 (29), 43 (81), 41 (51); 1,6-nonanediol (V): MS, *m/z* (%): M^+ 160 (0), 142 (18), 99 (46), 81 (89), 73 (58), 71 (13), 70 (27), 57 (31), 55 (100), 43 (48); (*E*)-coniferyl alcohol (VI): MS, *m/z* (%): M^+ 180 (86), 152 (12), 147 (15), 137 (100), 131 (15), 124 (47), 119 (28), 109 (13), 91 (33), 77 (25), 65 (12), 55 (17), 44 (17).

Wind Tunnel Experiment The effect of ME-feeding by *B. carambolae* males in the sexual attraction of conspecific females and males was assayed in a wind tunnel as described by Hee and Tan (1998). A wire mesh cage (15×9 cm diameter) containing 15 sexually mature ME-fed males was used as an attractant source and was placed at the upwind end of the tunnel. Fifteen sexually mature females (or males) were released from the downwind end and assayed for their behavioral response to the attractant. The numbers of respondents performing zigzag anemotactic flight over at least 50 cm to the source of attraction, as defined by Kennedy and Marsh (1974), were noted. Light intensity (average of up-, middle-, and downwind sections) inside the plastic tunnel was measured with a Lutron LX-101 lux meter at the beginning of each 10-min assay. The experiment was conducted from 18:00 to 20:00 hours or until the light intensity approximated 0 lx. Air flow in the wind tunnel was regulated at 15–17 cm/sec. The wind tunnel received natural daylight from the north.

To ascertain the effect of ME feeding by males in the attraction of *B. carambolae*, females were assayed to the ME-fed male source at 1 and 3 DPT, while the ME-deprived male source was used as control. To study the effect of ME feeding in the male-to-male interaction, untreated males were assayed to the ME-fed male source, while the attraction of untreated males to the untreated male source and ME-fed males to the untreated male source were used as negative and positive controls, respectively. Each treatment was replicated between four and ten times with different cohorts on different days. The proportion of responding flies over the total number tested (N=15) was transformed to a modified arcsine square root (Anscombe 1948) before being subjected to one-way variance analysis. Mean responses of females and males at each time interval to various male sources were separated by using least significant difference (LSD) test at P=0.05.

Field Cage Mating Experiment To determine the time lapsed between ME-feeding and heightened mating success against ME-deprived males, ME-fed males of 1, 2, and 3 DPT were used. A major host tree, starfruit (*Averrhoa carambola* L.) (canopy: 2.0 m height and 0.5 m width), was placed in the center of the field cage (2.1×2.1×2.1 m) to provide shade and resting sites for the flies upon release. A hundred and fifty each of ME-fed and ME-deprived males and females were released into the field cage at least 4 hr before courtship began to enable acclimatization. Males were released approximately 1 hr earlier than females to allow time for territorial establishment (McInnis et al. 1999).

Approximately 2 hr after dusk (sunset at ca 19:30 hours), by using red light, mating pairs were individually collected into separate specimen vials, and each male was visually inspected for color mark on the thorax to confirm if it was a ME-fed or -deprived individual. Results were subjected to Student *t*-test for paired samples at P=0.05.

Results

Collection of Male Volatile Emission In the aeration chamber, the male flies mostly rested with minimal locomotive activity during the pre-courtship period (16:00–18:00 hours). At dusk, a decrease in the light intensity to below 1,000 lx stimulated the initiation of excited locomotive activities, i.e., rapid walking and erratic flying by males. When the light intensity decreased to below 600 lx, males were observed to exhibit more erratic flying with intermittent wing fanning and anal-beating behavior (a means of pheromone dispersal in most *Bactrocera* spp.) (Kuba and Sokei 1988). This was followed by mounting attempts on other males in close proximity. As time elapsed and with a further decrease in light intensity, more males engaged in wing fanning, resulting in a visible whitish pheromone smoke within the enclosed glass chamber. As soon as the sun set and the light intensity decreased to below 2 lx, most males became inactive by resuming the resting posture.

Gas chromatographic analysis on the eluate from the Tenax TA traps revealed the presence of a ME-derived compound, CF (VI), along with five endogenous pheromonal compounds [*N*-(3-methylbutyl) acetamide (I), ethyl benzoate (II), benzamide (III), 6-oxo-1-nonanol (IV), and 1,6-nonanediol (V)] during the courtship period (18:00–20:00 hours, 2–2,000 lx; Fig. 1b) (a broad peak at a retention time of 6.30 min, which appeared in varying quantities, is unknown). These compounds were absent before the courtship period (16:00–18:00 hours, >2,000 lx; Fig. 1a) and after sunset (20:00–22:00 hours, <2 lx; Fig. 1c).

Wind Tunnel Experiment In the wind tunnel, the attraction of *B. carambolae* females and males to the conspecific males generally increased concomitant to decreasing light intensity (Figs. 2 and 3). In the female-to-male attraction, females' attraction to the untreated control males increased gradually and peaked (33%) at 19:30 hr (ca 18 lx), followed by a drastic decrease (4%) at 19:45 hours, when the light intensity dropped to ca 3 lx ($F_{7,72}$=2.47, P=0.025) (Fig. 2). ME feeding by males, both at 1 and 3 DPT, increased the overall attraction of females ($F_{7,40}$=18.23, P=1.25×10^{-10}; $F_{7,48}$=36.76, P=3.40×10^{-17}) (Fig. 2). However, peak attraction was observed at least 1/2 hr earlier at 3 DPT (18:45–19:00 hr) compared to 1 DPT (19:15–19:30 hours) (Fig. 2).

When compared across treatments, there was no significant difference in the female attraction to various male sources from 18:00 to 18:15 hours (Fig. 2). However, males of 3 DPT attracted more females than the other two treatments beginning from 18:30 to 18:45 hours (P<0.05) (Fig. 2). At 19:00 hours, males of 1 DPT attracted more females than untreated males, but the attraction was lower than that of the 3 DPT males (P<0.05) (Fig. 2). While the attraction of females to the 3 DPT males seemed to decline at 19:15 hours, it was not significantly different from that of the 1 DPT males (P=0.05), which began to attract more females from 19:30 to 19:45 hours than the other two treatments (P<0.05) (Fig. 2).

In general, even though there was a temporal effect on the male-to-male interaction, the average response of untreated males to the untreated male source (=negative control) was low (ca 7%), with a 12% peak at 19:30 hours ($F_{7,32}$=2.51, P=0.036) (Fig. 3). Males fed with ME were still responding to the untreated male source (=positive control) ($F_{7,24}$=3.16, P=0.016) with the peak of attraction (12%) similar to that of the untreated males, but the average response was also low (ca 3%). There was no significant difference between these two controls throughout the assay time except at 19:00 hours (P<0.05) (Fig. 3). However, when the ME-fed males were used as an attraction source, there was an increase in the

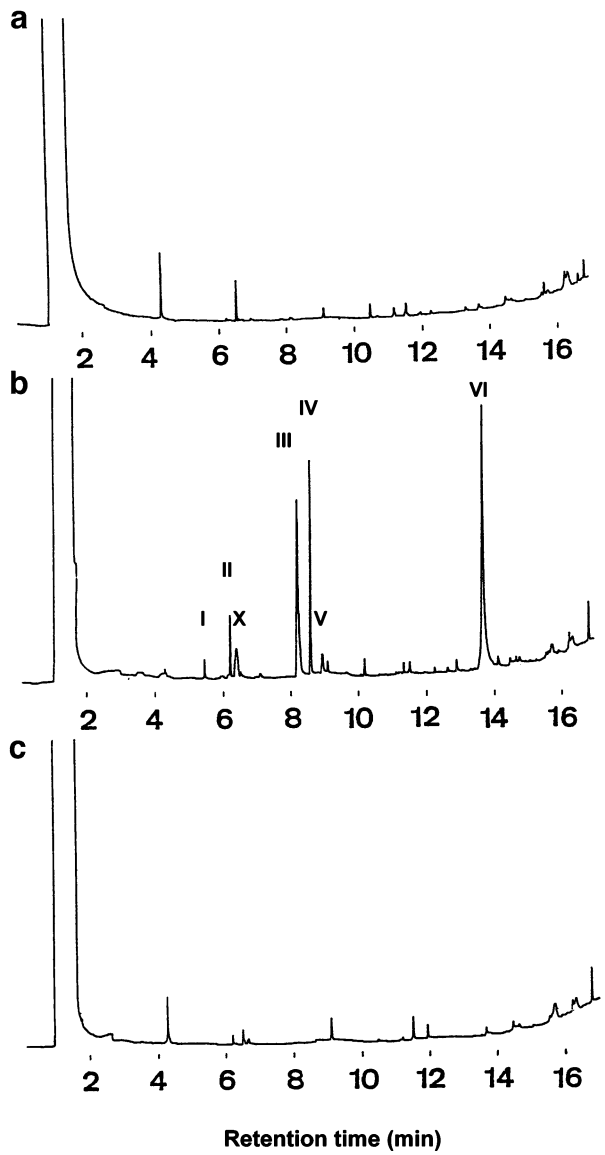


Fig. 1 Gas chromatograms of volatile emission of methyl eugenol-fed *Bactrocera carambolae* males, aerated at **a** 16:00–18:00 hours (before sunset), **b** 18:00–20:00 hours (during sunset), and **c** 20:00–22:00 hours (after sunset). Compound I = *N*-(3-methylbutyl) acetamide, II = ethyl benzoate, III = benzamide, IV = 6-oxo-1-nonanol, V = 1,6-nonanediol, and VI = (*E*)-coniferyl alcohol. Compound X, eluted immediately after compound II, is unknown

overall male-to-male attraction ($F_{7,24}$=6.49, P<0.001), with a peak of 68.3% at 19:15 hours (Fig. 3). When each of the time components was analyzed, males' response to the ME-fed male source was always higher than to the other two treatments (P<0.05), except at the beginning (18:15 hours) and at the end (19:45 hours) of the bioassay (P>0.05) (Fig. 3).

Field Cage Mating Experiment In the field cage, there were no significant differences in male mating success between the ME-fed and the ME-deprived males at 1 DPT (t=0.82, df=11) and 2 DPT(t=1.64, df=4) (P>0.05; Table 1). However, ME-fed males at 3 DPT were more competitive than ME-deprived males in mating (t=7.26, df=8; P<0.001).

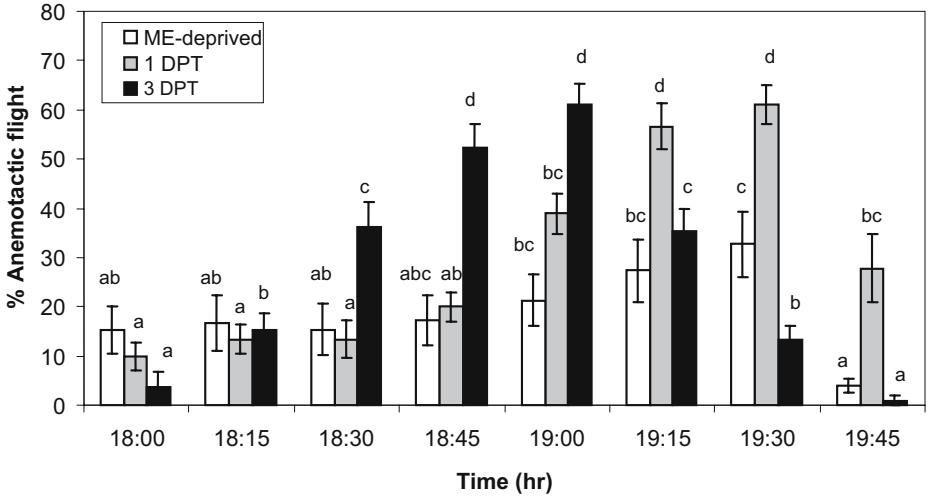


Fig. 2 The attraction of *Bactrocera carambolae* virgin females to various live male sources: (*a*) methyl eugenol (ME)-deprived, (*b*) 1-d posttreatment of ME (1 DPT), and (*c*) 3-d posttreatment of ME (3 DPT), during courtship period in wind tunnel assays. *Bars* (=standard errors) followed by *different alphabets* within the same treatment are significantly different at $P=0.05$ (LSD test)

ME-fed males initiated courtship behavior earlier than the ME-deprived males, consistent with the observation in the wind tunnel assays. The ME-fed males were evidently more attractive to females—in that a female would extrude her ovipositor toward a ME-fed male, a sexual acceptance stance in the tephritids, even though the male was still *in copula*. ME-deprived males were often seen aggregated around a ME-fed male, who was either calling (Fig. 4a) or mating (Fig. 4b), and they fed on the anal region of the ME-fed

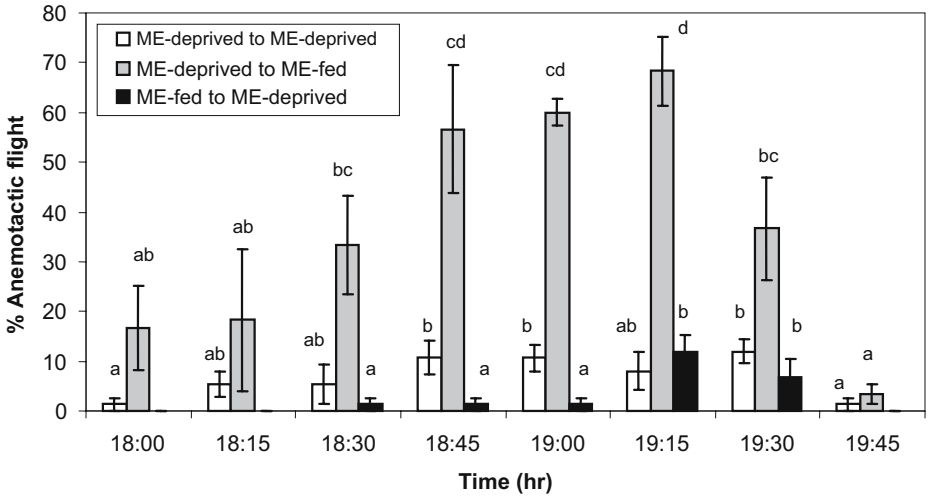


Fig. 3 The attraction of *Bactrocera carambolae* virgin males to various live male sources: (*a*) methyl eugenol (ME)-deprived to ME-deprived, (*b*) ME-deprived to ME-fed, and (*c*) ME-fed to ME-deprived males during courtship period in wind tunnel assays. ME-fed males used were at 3-d posttreatment of ME. *Bars* (=standard errors) followed by *different alphabets* within the same treatment are significantly different at $P=0.05$ (LSD test)

Table 1 Mating success of *B. carambolae* males at 1, 2, and 3 d posttreatment (DPT) of methyl eugenol (ME)

Treatment	Number of Replicates (*N*)*	Mean±SE		*P* Values
		ME-fed	ME-deprived	
1 DPT	12	35.8+2.8	32.5±3.6	0.21
2 DPT	5	26.8±5.0	20.0±8.3	0.09
3 DPT	9	49.9±1.3	29.8±1.9	4.4×10^{-5}

*300 flies (100 each of virgin females, ME-fed and ME-deprived males) in each replicate

male. Anal secretion collected from the ME-fed males contained compound CF in addition to the endogenous pheromonal compounds of a *B. carambolae* male.

Discussion

The pharmacophagy of ME by males of *B. carambolae* has yielded three major findings that have important implications in the chemical ecology of this species: (a) ME was biotransformed (almost immediately) into compound CF, which was temporarily stored in the rectal gland and subsequently released at dusk by the ME-fed males; (b) ME-fed males showed an earlier onset of courtship behavior and were perceived by conspecific females to be more attractive than ME-deprived males, at a time lapse of 3 DPT, as evidenced by the results of the wind tunnel bioassays and field cage trials; and (c) ME-fed males induced an aggregation behavior in conspecifc males during the courtship period, and the aggregated males fed on the anal secretion of the ME-fed males that contained CF together with the male endogenous pheromonal compounds.

B. carambolae, being a dusk-mating species, has its courtship behavior confined to periods between 18:00 to 20:00 hours (photoperiod 12 L:12 D). The fact that males: (a)

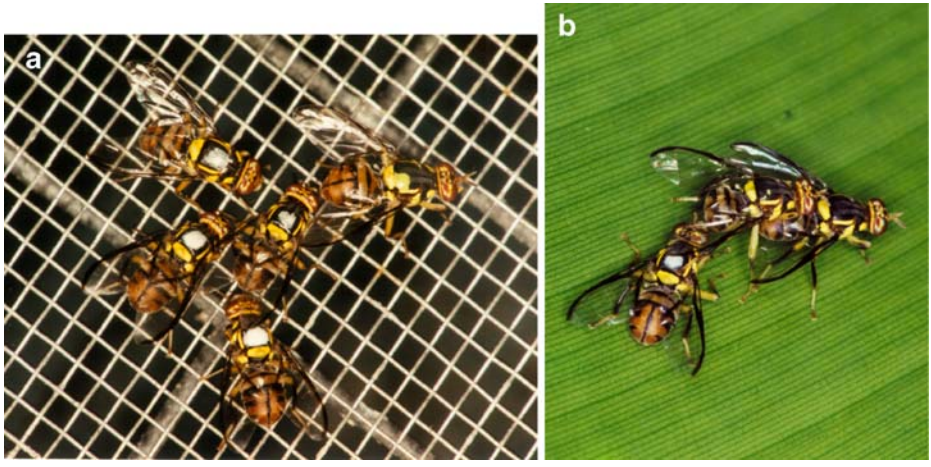


Fig. 4 Methyl eugenol (ME)-deprived *B. carambolae* male(s) (thorax marked with white paint) feeding on the anal secretion of a ME-fed male (**a**) at calling (*top most right side*) and (**b**) at mating in a field cage assay

responded maximally to and fed on ME during the first few hours of photophase (Wee and Tan 2000; Wee et al. 2002); (b) accumulated the biotransformed phenylpropanoid, i.e., CF, within 15 min after ME consumption and stored it in the rectal gland for up to 2 wk (Wee and Tan 2007); and (c) subsequently released CF that is attractive to both conspecific males and females during courtship, supports the hypothesis that ME plays a central and mediating role in the sexual communication of this species. In addition, the detection of ME metabolites in the rectal glands of 83–100% wild males of ME-sensitive *Bactrocera* species indirectly indicates the importance of ME foraging activities and role of ME in the chemical ecology of the ME-sensitive *Bactrocera* species (Tan and Nishida 1998; Wee and Tan 2001).

Interspecific variation is evident in the time lapse between ME feeding and the heightened mating success of *B. carambolae* compared to *B. dorsalis*, a closely related sympatric sibling species of the *dorsalis* complex. ME-fed males have an earlier onset of mating ritual (Hee and Tan 1998) and a higher mating success at just 1 d after feeding on ME (Tan and Nishida 1996, 1998) and continue to enjoy this mating advantage for as long as 3 wk (Shelly and Dewire 1994). Although ME-fed *B. carambolae* males showed a significant sexual attraction at 1 DPT when compared with the ME-deprived males, significant mating success did not occur at 1 or 2 DPT but only at 3 DPT. A similar phenomenon has also been observed for *B. philippinensis*, another *B. dorsalis* sibling species, in which males only had a mating advantage at 3–5 d postfeeding on ME (Shelly et al. 1996). This discrepancy may be explained by a comparison of the quantitative temporal accumulation of ME metabolite (s) in the rectal glands of *B. carambolae* and *B. dorsalis* after consumption of a standard quantity of ME. In both sibling species, ME metabolite(s) increase gradually with time, but with a significant decrease in the accumulation rate (defined as accumulation after partial release of the metabolite(s) during the courtship period) that is only evident at 3 d post-ME feeding for *B. carambolae* compared with 1 d in *B. dorsalis* males (Wee and Tan 2007).

Aggregation behavior or lek formation is commonly observed in the Oriental fruit fly, *B. dorsalis*, and Mediterranean fruit fly, *Ceratitis capitata*. Numerous studies have been attempted to ascertain the role of leks in mating systems of tephritids (Kaspi and Yuval 1999; Shelly and Kaneshiro 1991; Shelly 2000b, 2001b). Generally, it is hypothesized that lekking provides a communal display area where males congregate, thus attracting and courting females and leading to copulation (Emlen and Oring 1977). In *B. carambolae*, the lek behavior was not evidently displayed in the male-to-male attractancy results. However, after feeding on ME, ME-fed males attracted more conspecific males in the wind tunnel, and the attracted males apparently searched for and fed on the anal region of the ME-fed males (as observed in the field cage experiment). CF acts as an aggregation pheromone in male–male communication during the courtship period (evident in the chemical analysis of the anal secretion). The aggregation behavior and anal secretion feeding by ME-deprived males happened just before and during the courtship period. Such behavior may have diverted their attention from mating and contributed partly to the higher mating success in the ME-fed males.

Coniferyl alcohol shows some degree of allomonal activity in deterring vertebrate predators (Nishida and Fukami 1990). However, its role may be limited to intraspecific interaction (pheromonal) in *B. carambolae*. A previous study has shown that with attainment of sexual maturity and the production of endogenous pheromonal compounds, *B. carambolae* males develop an inherent antipredation mechanism against gecko predation. It also shows that having an additional compound, i.e., CF, in the rectal gland of ME-fed *B. carambolae* contributes little/negligible deterrent effect against gecko predation when compared with sexually mature but ME-deprived males (Wee and Tan 2005b).

Methyl eugenol is carcinogenic to vertebrates (Miller et al. 1983; Wee and Tan 2001). ME-attracted tephritids detoxify ME by simple oxidative processes and biotransform it to by-product(s) that are stored in the rectal gland. ME-attracted tephritid male flies usually appear sluggish immediately after ME consumption compared to unfed males (Shelly and Dewire 1994; Wee and Tan 2007). This sluggishness is more obvious in *B. carambolae* than in *B. dorsalis*. It was induced in the former species even after consumption of a minute quantity of ME and eventually caused approximately 10% mortality at 12-d posttreatment (Wee and Tan 2007).

Males of *B. carambolae*: (a) synthesize large amounts of endogenous pheromonal components (the average peak production of the major component, 6-oxo-1-nonanol, was 26.1±12.5 μg/male (Wee and Tan 2005a); (b) produce benzamide that is detected in varying quantities in the aeration products and in the male rectal extracts; and (c) at the same time, engage in ME foraging activity. However, when compared with its sibling species, *B. dorsalis* utilizes an external precursor, i.e., ME, and biotransforms it into chemicals that offer reproductive advantage in mating competition and protection against vertebrate predator (Tan and Nishida 1996, 1998; Hee and Tan 1998; Wee and Tan 2001), whereas *B. carambolae* males spend more metabolic energy. Enzymes are needed to catalyze the various metabolic reactions required to synthesize each of the endogenously synthesized compounds. Resources are used that might otherwise be used for growth or reproduction (Pasteels et al. 1983). From an evolutionary perspective, this suggests that *B. carambolae* is probably less advanced than *B. dorsalis* in species speciation within the *dorsalis* complex.

Acknowledgements This work was funded by Malaysian IRPA grant (NO: 01-02-05-9007) and partly supported by the International Atomic Energy Agency (Contract No. 10570) given to K. H. Tan. We thank Andy R. Gibb (HortResearch, New Zealand) for his comments.

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Introduction

Allelopathic compounds can be either actively released by a plant itself (Ridenour and Callaway 2001; Seal et al. 2004) or passively produced during the decomposition process of plant residues (Blum et al. 1999; Tseng et al. 2003; Bonanomi et al. 2006). Downed logs act as nurse or preferred sites for forest seed germination. Although making up only a small percentage of the microsites, they account for a disproportionate percentage of established seedlings. This phenomenon is well documented for a variety of forest types, including temperate forests (Santiago 2000; Takahashi et al. 2000; Brang et al. 2003; Liao et al. 2003; Narukawa et al. 2003) and rain forests (Christy and Mack 1984; Lusk 1995). Different hypotheses for the advantages of downed log seedbeds for establishing tree seedlings have been proposed: high water-holding capacity results in reduced moisture stress (Place 1955; Tubbs 1996), early snow melts on the elevated substratum and can cause a longer growing season (Harmon et al. 1986), infection with pathogenic fungi is reduced (Cheng and Igarashi 1987; O'Hanlon-Manners and Kotanen 2004), impact of competing vegetation is decreased (McKee et al. 1982; Hiura et al. 1996), standing water excludes seedlings from the soil (McKee et al. 1982), phytotoxic compounds released by ground vegetation restrict seedlings to logs (del Moral and Cates 1971). There is no literature describing allelopathy of conifer tree seedlings growing on logs.

The Yuanyang Lake (YYL) Nature Preserve is one of the Long-Term Ecological Research sites in Taiwan. *Chamaecyparis obtusa* var. *formosana* and broadleaf trees coexist in the forest at YYL (Chou et al., 2000). The forest is relatively mature, and *C. obtusa* var. *formosana* is regenerating and sustainable as the dominant species in that area (Chen and Yang 1996; Chou et al. 2000; Liao et al. 2003). *C. obtusa* var. *formosana* is a shade-intolerant species (Chiu et al. 1995; Lo-Cho et al. 1999) and the only species creating both canopy gaps and downed logs in the forest. Those gaps are filled mostly with the seedlings of *C. obtusa* var. *formosana* (Liao et al. 2003). Seedlings establish themselves on downed logs soon after the logs fall. Their survival rate is high on newly downed logs (Chang et al. 2001; Liao et al. 2003). There are more seedlings of *C. obtusa* var. *formosana* than those of broadleaf trees germinating on the newly downed logs (Liao et al. 2003).

Based on a field investigation and previous research, we hypothesized that the bark of newly downed logs possessing allelopathic potential provides a habitat especially suitable for germination and subsequent growth of *C. obtusa* var. *formosana* seedlings. Six kinds of seeds collected from YYL, and along with lettuce and one invasive weed, *Bidens pilosa*, were germinated on the bark of *C. obtusa* var. *formosana*. Their percent germination, survival rates, root length, fresh weight, chlorophyll contents, and soluble protein contents were compared to evaluate the phytotoxic effect of the log bark. Bioassay of a phytotoxic compound isolated from the bark was conducted.

Materials and Methods

Study Site The study site was located in the YYL, on Chi-lan Mountain (24°35′ N, 121°24′ E) in the northeastern part of Taiwan, between 1,650 and 2,432 m altitude. Yanuyang Lake has a maximal depth of 4 m, an area of 3.6 ha. Annual precipitation, mostly contributed by monsoons and typhoons, is between 3,000 and 4,000 mm (Hwang et al. 1996; Wu et al. 2001). Heavy cloud and fog are so frequent that solar radiation is remarkably reduced. Daily solar radiation data at the YYL ranged from 0 to 25 MJ/m^2/day, and the mean air temperatures ranged from 5 to 17.5°C (Hwang et al. 1996). The forest soils are generally

shallow (<60 cm) with granite bedrock underground (Chen and Chiu 2000). The thick organic layer, probably derived from peat, directly covers the primarily weathered bedrock. Poor drainage, originating from the clay or silty clay mineral layer beneath the organic layer, limits the downward movement of minerals, and consequently, retards the development of soil profile (Chiu et al. 1999; Wu et al. 2001). Shallow soil, frequent typhoons, and steep slopes may contribute to the large number of deaths and fallen trees of *C. obtusa* var. *formosana* in the YYL forest community (Lo-Cho et al. 1989; Chang et al. 2001).

The vegetation consists of coniferous forest, hardwood forest, pteridophytes, epiphytes, such as mosses and liverworts, grassland species, and several aquatic plants. Floristic analysis indicated that the forest could be divided into two communities, namely, a coniferous–hardwood community and a swamp or early succession community (Chou et al. 2000). Within the forest, *C. obtusa* var. *formosana* is the dominant species and occupies canopy layer above 20 m height. *Rhododendron formosanum* is predominant among the trees below 10 m height around the lakeside (Chou et al. 2000). According to pollen analysis (Chen and Wu 1999), floristic analysis, and census of seedlings (Chou et al. 2000; Liao et al. 2003), the forest in YYL has existed for at least 4,000 yrs, and it has been speculated that the vegetation, floristic composition, and canopy structure of this forest will not change in the near future.

Seedling Growth Tests Seeds of all species except lettuce and *B. pilosa* were collected in YYL before seed dispersal during the autumn of 2004 and 2005. The seeds of two dominant species (*C. obtusa* var. *formosana* and *R. formosanum*), one grassland species (*Miscanthus transmorrisonensis*), two kinds of broadleaf trees (*Barthea formosana* and *Pieris taiwanensis*), and one conifer tree C. *formosensis* were collected. The bark of new *C. obtusa* var. *formosana*-downed logs was collected for germination tests. After seeds were immersed in water for 3 d, except lettuce, *B. pilosa*, and *M. transmorrisonensis* immersed for 2 hr, all seeds were separately sown on the bark (7×8×0.5 cm) saturated with 50 ml of distilled water or onto one stack of 10 pieces of circle filter paper (90 mm, Advantec Toyo, Japan) saturated with 50 ml of 1.5 mM salicylic acid solution (2-hydroxybenzoic acid; purity ≥99%, Merck) or with distilled water as control. The number of seeds sown on bark or filter paper varied with seed size. Lettuce, *B. pilosa*, *C. obtusa* var. *formosana*, and C. *formosensis* all had 50 seeds/per treatment, whereas *R. formosanum*, *M. transmorrisonensis*, *B. formosana*, and *P. taiwanensis* all had 100 seeds/per treatment for each of 3 reps.

The dose response curve for inhibition of lettuce germination by salicylic acid showed that 1.5 mM was the concentration that completely inhibited lettuce germination similar to that on the bark. Therefore, the concentration of salicylic acid at 1.5 mM was chosen and applied to filter paper. Each treatment was put in one transparent glass container (30π× 3.0 cm) sealed by transparent plastic film (Nan Ya Plastics Industrial Co.) to prevent drying out. All seeds were incubated in a growth chamber with 22±1°C, 16:8 hr day is to night ratio, and illumination of 85 μEm^{-2}. Average percent germination, survival rate, fresh weight, root length, soluble protein content, and chlorophyll content were measured after 7 wk, except those of lettuce and *B. pilosa*, which were measured after 1 wk. Whole seedlings were homogenized in extract medium that contained 50 mM Hepes buffer (pH 7.5), 10 mM 2-mercaptoethanol, 1% polyvinylpyrrolidone (PVP), 2 mM ethylenediaminetetraacetic acid (EDTA)-Na_2, 1 mM $MnCl_2$, 1 mM $MgCl_2$. Extracts were centrifuged at 18,000×*g* for 15 min, and the supernatant was used for determination of protein by Coomassie Blue spectrophotometric assay (Spector 1978) by using dye regent purchased from Bio-Rad, Laboratories, Inc. with bovine serum albumin as the standard. Chlorophyll was determined in 96% ethanol using the absorption coefficients of Wintermans and De Mots (1965). Data were expressed as the ratio of the treatment value

divided by the control in which seeds were germinated in distilled water for making comparisons among various species.

Extraction and Isolation of Phytotoxic Compound Bioassay-guided fractionation and purification based on phytotoxicity against lettuce were conducted. For each separation, the obtained fractions were concentrated and dried under *vacuo* at 40±2°C and then bioassayed against lettuce according to Tseng et al. (2003). The pure or crude compounds were dissolved in spectroscopic grade ethyl acetate to various concentrations and spread onto silica gel thin layer chromatography (TLC) sheets. The ethyl acetate was allowed to evaporate completely in a laminar flow hood. Before bioassay, the TLC sheets were placed into Petri dishes and moistened with distilled water. Test seeds were soaked in distilled water and then placed onto the TLC sheets containing the pure compound or onto control sheets. Petri dishes were sealed with parafilm "M" (American National Can™) and placed in an incubator at 25°C for 48 hr in the dark. Two and a half kilograms of dried chipped bark were extracted with 20 l MeOH at room temperature for 10 d. The extract was evaporated *in vacuo* to yield a residue that was then suspended in H_2O (200 ml) and partitioned with ethyl acetate (500 ml×3). The combined ethyl acetate layer afforded a brown syrup (ca 180 g) that was subsequently chromatographed over silica gel (Merk 70-230, 230-400 mesh, ASTM) by using a hexane/ethyl acetate gradient solvent system. Eventually 22 fractions (F1-F22) were collected. F9–F10 showed the greatest inhibition of lettuce germination. The combination (2.9 g) of F9–F10 was further purified by silica gel column eluted with 0–100% ethyl acetate in dichloromethane, and 13 fractions were collected (F′1–F′13). F′3–F′5 were combined and purified by high-performance liquid chromatography (HPLC) [Merk Lichro CART 250-10 Cat. 1.50179 Lichrosorb Si 60 (7 μm)] and Sephadex LH 20. About 180 mg pure compound with phytotoxicity against lettuce were isolated and identified by spectroscopic identification with an infrared (IR) (Perkin-Elmer 983G Spectrophotometer), proton (^{1}H) and carbon nuclear magnetic resonance (^{13}C NMR) (Varian Unity Plus 400 spectrometer), gas chromatography/mass spectroscopy (GC/MS) (Agilent 6890 N Gas Chromatography /Agilent 5973 N Mass Spectrometer). The GC/MS analyses were carried out by using an Agilent 6890 N GC system coupled to an Agilent 5973 mass selective detector (MSD). The GC column used was an HP-5 MS capillary column (30 m×0.2 mm I.D., 0.33 μm film thickness). The GC was operated in the splitless mode when performing injections with the aid of an Agilent 7683 series autosampler. An injection port temperature at 280°C was used. For the isolated compound and salicylic acid standard analysis, the column temperature was programmed from 80 to 280°C at 15°C/min with a 7-min hold at 240°C and a 4.67-min hold at 280°C. Helium of 99.9% purity was used as the carrier gas at a flow-rate of 1 ml/min. The splitless inlet liner is a deactivated 1/pk single-taper packed with glass wool (Agilent; part number: 5062-3587). Transfer line temperature was 280°C, and a 70-eV electron-impact ionization source was held at 280°C.

Statistics Means were compared by using one-way analysis of variance (ANOVA), Tukey's test and Pearson correlation analysis in SAS statistical software (SAS 2004).

Results

Establishment of Seedlings on Bark of C. obtusa var. formosana All eight seed species were able to germinate on the bark. The germination percentages among five species, lettuce, *M. transmorrisonensis, P. taiwanensis, B. formosana*, and *C. obtusa* var. *formosana* were not significantly different (Fig. 1). *R. formosanum* had the highest germination rate

and *B. pilosa* the lowest. Seedlings of lettuce, *B. pilosa*, and *C. formosensis* were stunted, turned yellow, and died on the bark before harvest. The seedling survival rates of *C. obtusa* var. *formosana* and *R. formosanum* were obviously higher than other species (F=34.08, P<0.001). Differences in seedling survival rate among *M. transmorrisonensis*, *P. taiwanensis*, and *B. formosana* were not significant; however, *M. transmorrisonensis* could barely develop its radicle roots despite normal growth of shoots on the bark (Fig. 2). Fresh weight and root length of *C. obtusa* var. *formosana* and *R. formosanum* were also significantly higher than other species (Fig. 2). In addition, there was a significant positive correlation

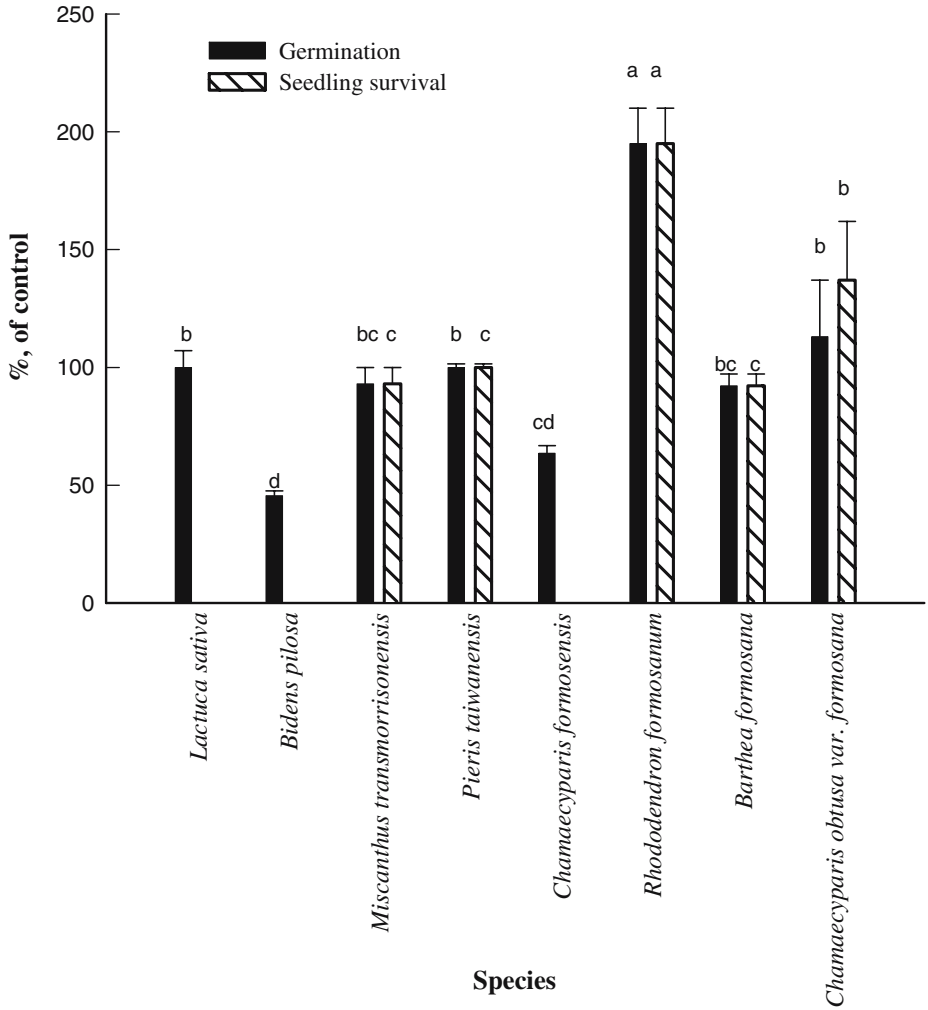


Fig. 1 Germination and seedling survival of different species on bark of *C. obtusa* var. *formosana*. *Different letters above the standard error bars* designate significant differences at the 0.05 level by Tukey's test. The absolute control average values of percent germination are as following: *L. sativa* (86.1%), *B. pilosa* (77.7%), *M. transmorrisonensis* (90.8%), *P. taiwanensis* (91.4%), *C. formosensis* (49.5%), *R. formosanum* (28.0%), *B. formosana* (23.0%), *C. obtusa* var. *formosana* (64.9%). Those survival rates are the same as percent germination for most species except *C. obtusa* var. *formosana*, which was 57.7% because several *C. obtusa* var. *formosana* seedlings were infected by fungi

between fresh weight and root length of *M. transmorrisonensis*, *P. taiwanensis*, *R. formosanum*, *B. formosana*, and *C. obtusa* var. *formosana* (Pearson correlation, r=0.97, P<0.01), indicating that root length of these species was inhibited more than their fresh weight on the bark (Fig. 2).

Soluble Protein and Chlorophyll Contents of Seedlings on Bark Soluble protein contents were not significantly different among those five species established on the bark, *M.*

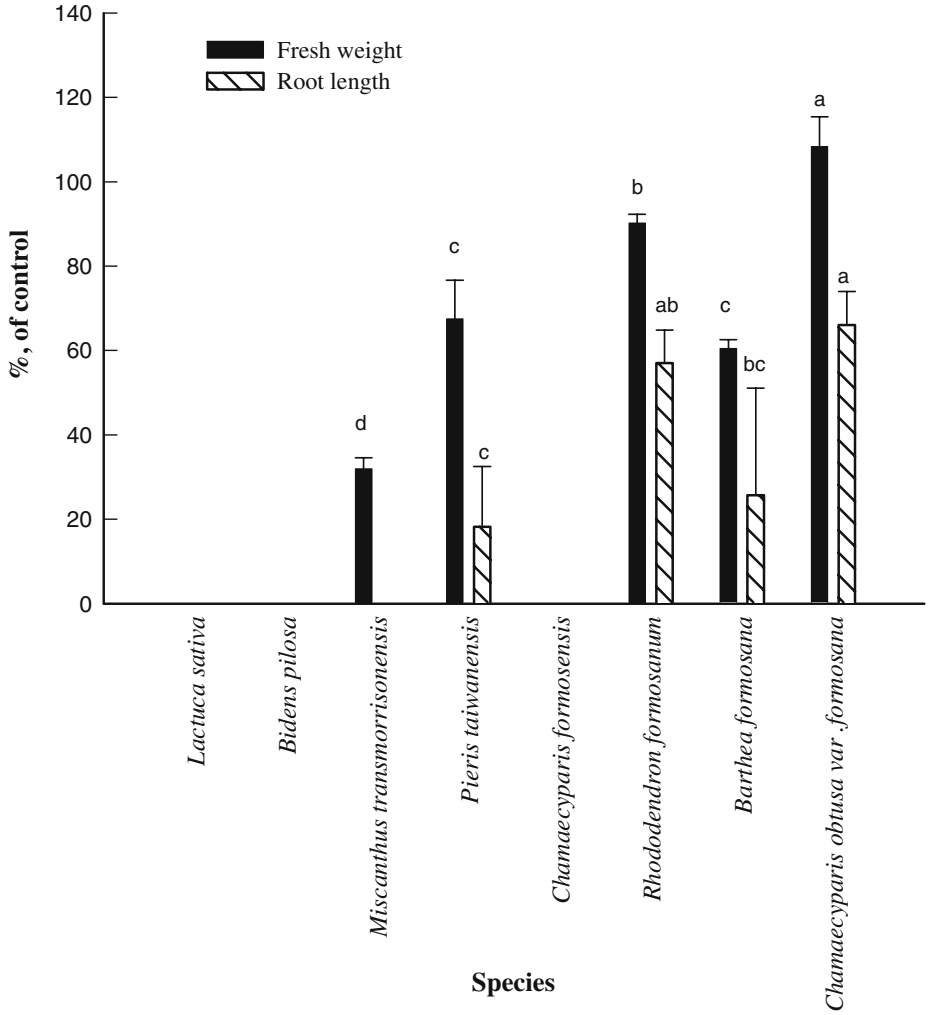


Fig. 2 Fresh weight and root length of different kinds of seedlings grew on bark of *C. obtusa* var. *formosana*. *Different letters above the standard error bars* designate significant differences at the 0.05 level by Tukey's test. The absolute control average values of fresh weight are as following: *L. sativa* (1.08×10^{-2} g), *B. pilosa* (0.84×10^{-2} g), *M. transmorrisonensis* (0.87×10^{-2} g), *P. taiwanensis* (0.34×10^{-2} g), *C. formosensis* (0.69×10^{-2} g), *R. formosanum* (0.25×10^{-2} g), *B. formosana* (0.09×10^{-2} g), *C. obtusa* var. *formosana* (1.41×10^{-2} g). Those of root length are as follows: *L. sativa* (60.7 cm), *B. pilosa* (36.1 cm), *M. transmorrisonensis* (136.0 cm), *P. taiwanensis* (42.6 cm), *C. formosensis* (29.7 cm), *R. formosanum* (20.2 cm), *B. formosana* (6.7 cm), *C. obtusa* var. *formosana* (46.1 cm)

transmorrisonensis, *P. taiwanensis*, *R. formosanum*, *B. formosana*, and *C. obtusa* var. *formosana* (Fig. 3). The chlorophyll contents of *R. formosanum* and *C. obtusa* var. *formosana* were higher than *M. transmorrisonensis*, *P. taiwanensis*, and *B. formosana* (F= 77.4, P<0.001, Fig. 3).

Isolation and Identification of Phytotoxic Compound from Bark About 180 mg pure compound with inhibitory effect on lettuce were isolated from crude methanolic extract of

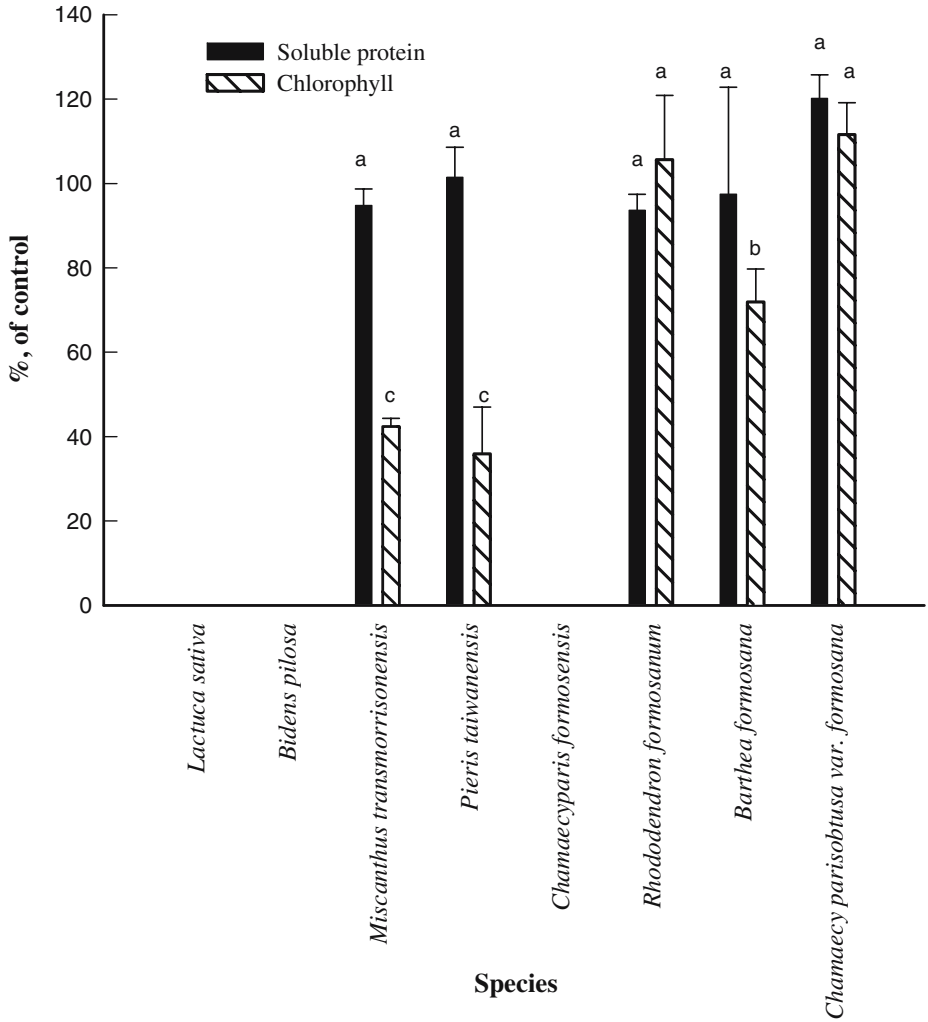


Fig. 3 Soluble protein and chlorophyll contents of seedlings grew on the bark of *C. obtusa* var. *formosana*. *Different letters above the standard error bars* designate significant differences at the 0.05 level by Tukey's test. The absolute control average values of soluble protein are as follows (μg/per one seedling): *L. sativa* (−), *B. pilosa* (106.7), *M. transmorrisonensis* (33.9), *P. taiwanensis* (27.2), *C. formosensis* (50.6), *R. formosanum* (18.8), *B. formosana* (7.4) , *C. obtusa* var. *formosana* (67.0). Those of chlorophyll are as follows (μg/per one seedling): *L. sativa* (−), *B. pilosa* (3.17), *M. transmorrisonensis* (2.05), *P. taiwanensis* (0.42), *C. formosensis* (1.20), *R. formosanum* (0.63), *B. formosana* (0.12), *C. obtusa* var. *formosana* (1.31). Note: "–" indicates that control data were not measured as *L. sativa* could not grow in test treatments

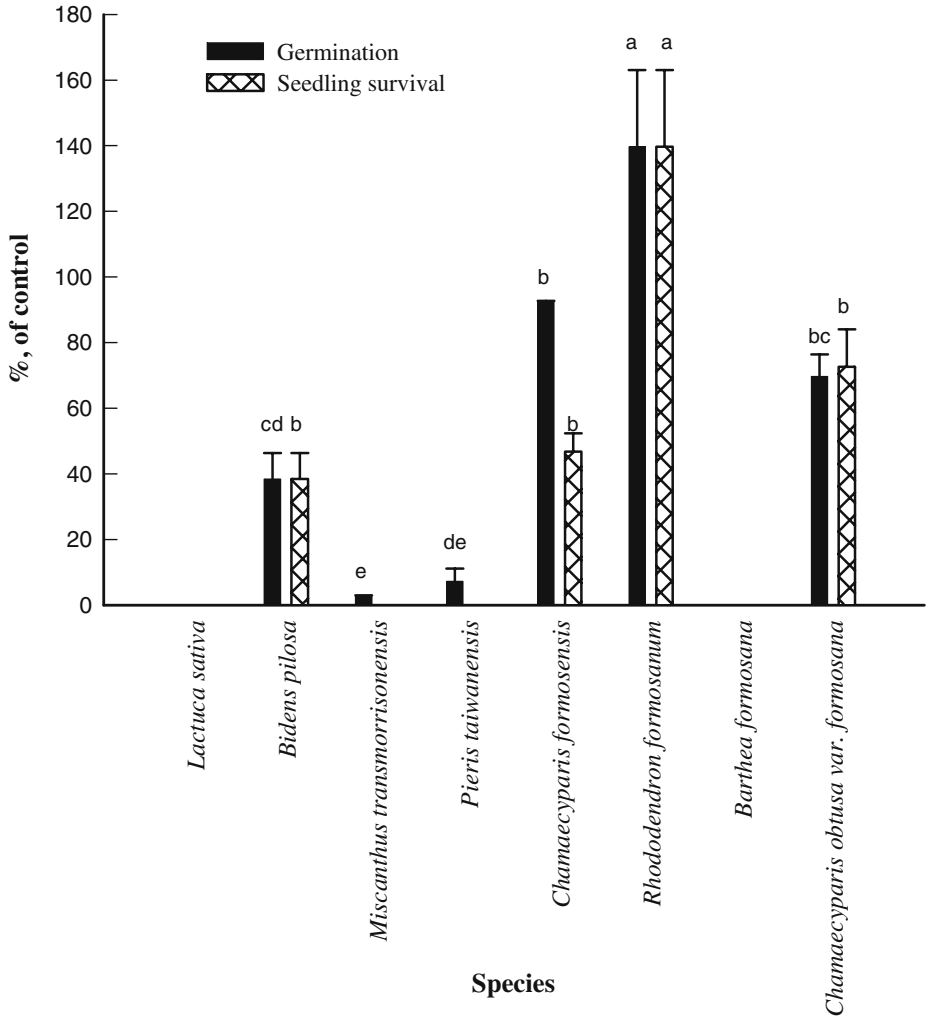


Fig. 4 Effect of salicylic acid on seed germination and seedling survival. *Different letters above the standard error bars* designate significant differences at the 0.05 level by Tukey's test. The absolute control average values were the same as in Fig. 1

the bark (2.5 kg) using bioassay-guided fractionation and purification. The IR spectrum of the isolated compound indicated the presence of a conjugated carboxylic [3,400~2,600 (broad band), 1,667 cm^{-1}], aromatic (1,613, 1,506 cm^{-1}) and hydroxyl (3,232 cm^{-1}) groups. The ^1H NMR showed signals of aromatic protons resonanced at δ_H 7.90 (1 H, dd, J=8.0, 1.6 Hz), 7.50 (1 H, ddd, J=8.4, 7.2, 1.6 Hz), 6.99 (1 H, dd, J=8.4, 1.2 Hz), and 6.91 (1 H, ddd, J=8.0, 7.2, 1.2 Hz), which indicated the *ortho*-substituted aromatic compound. The ^1H NMR data are similar to those of salicylic acid (Lampert et al. 1997; Singh et al. 2004; Emmler et al. 2004). Furthermore, the compound showed an identical retention time and mass spectrum with those of a salicylic acid standard in the GC/MS analysis. Those spectroscopic data were consistent with a structure of salicylic acid. The concentration of salicylic acid in the bark was estimated to be 72.0 μg g^{-1}.

Effect of Salicylic Acid on Seed Germination and Seedling Growth Six of the seeds, *M. transmorrisonensis*, *P. taiwanensis*, *B. pilosa*, *C. formosensis*, *R. formosanum*, and *C. obtusa* var. *formosana*, germinated on the paper with 1.5 mM salicylic acid. Among the six, only those seedlings of *B. pilosa*, *C. formosensis*, *R. formosanum*, and *C. obtusa* var. *formosana* survived on this substrate (Fig. 4). The germination percentages of *M. transmorrisonensis* and *P. taiwanensis* were low, their seedlings did not grow continuously, and they died before harvest. *C. formosensis*, *R. formosanum*, and *C. obtusa* var. *formosana* grew better than the other species, and their fresh weight, root length, and chlorophyll contents were significantly higher than *B. pilosa* (Figs. 5 and 6).

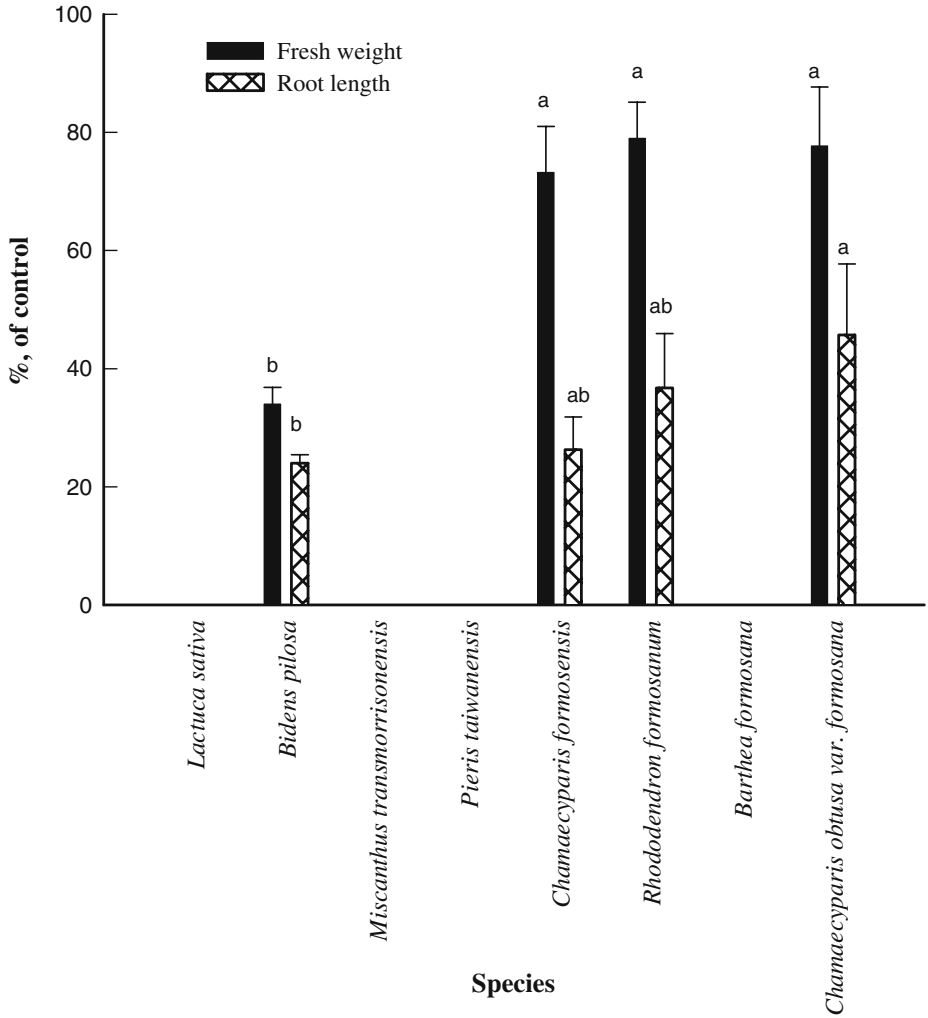


Fig. 5 Effect of salicylic acid on fresh weight and root length of different kinds of seedlings. *Different letters above the standard error bars* designate significant differences at the 0.05 level by Tukey's test. The absolute control average values were the same as in Fig. 2

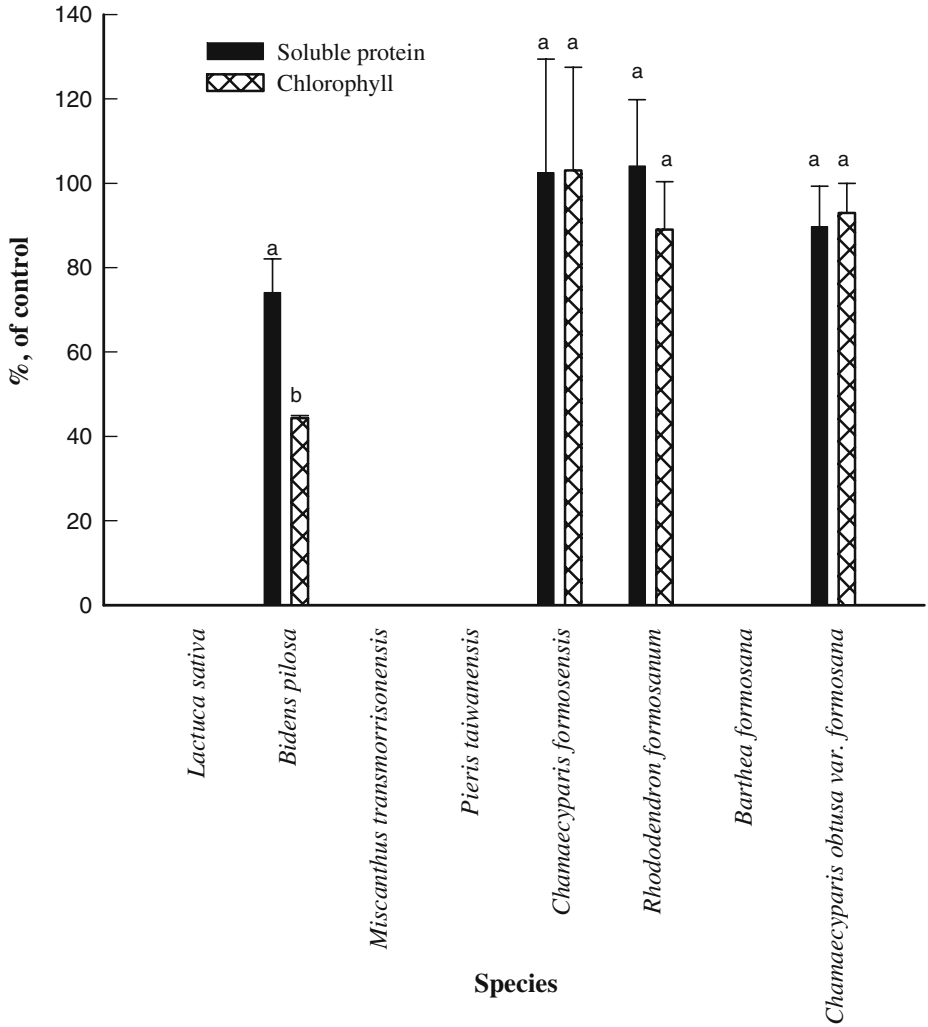


Fig. 6 Effect of salicylic acid on soluble protein and chlorophyll contents of seedlings. *Different letters above the standard error bars* designate significant differences at the 0.05 level by Tukey's test. The absolute control average values were the same as in Fig. 3

Discussion

The most drastic population changes of forest tree species occur during seed and seedling stages (Harper 1977; Harcombe 1987). Downed logs are important seedbeds for *C. obtusa* var. *formosana* due to higher seedling survival rate on them than on rocks and bare soils (Chang et al. 2001). The relative density of *C. obtusa* var. *formosana*, *R. formosanum*, and *B. formosana* measured on the sampled downed logs in YYL forest community were 59.8, 7.46, and 1.5%, respectively (Liao et al. 2003). In particular, nearly five times more *C. obtusa* var. *formosana* seedlings than other broadleaf seedlings were found on the newly downed logs (Liao et al. 2003). The survival rate, fresh weight, root length, and chlorophyll contents of *C. obtusa* var. *formosana* and *R. formosanum* seedlings grown on the bark were

significantly higher than those of *M. transmorrisonensis*, *P. taiwanensis*, and *B. formosana*. Root growth is important for successful establishment of seedlings on logs. Roots can absorb nutrients, penetrate into the clefts of bark, and prevent seedlings from washing away by rain. Bryophytes generally cover downed logs after trees fall. A favorable microhabitat is eventually created for tree seedlings due to the growth of bryophytes, organic matter deposition, bark decay, decayed wood, and humid weather. The layer of organic mater on logs allows root penetration of *C. obstusa* var. *formosana* and other broadleaf species.

Our results are consistent with the census data from the YYL forest (Liao et al. 2003). *C. obtusa* var. *formosana* and *R. formosanum* seedlings established on the downed logs had higher relative densities than did the seedlings of *B. formosana* and *M. transmorrisonensis*, *P. taiwanensis*, and *C. formosensis*, which were either not found on the logs or very few of them were established on the seedbeds. Lettuce and *B. pilosa*, which were not found in the YYL forest, were able to germinate but did not grow on the bark. These results suggest that the bark of *C. obtusa* var. *formosana* with its phytotoxic effect on other species might function as a regeneration niche favoring seedlings of *C. obtusa* var. *formosana* and *R. formosanum*.

To help understand the mechanism of toxicity, a compound was isolated and identified as salicylic acid. Salicylic acid had been reported as an allelopathic chemical and has been isolated during decomposition of plant residues (Chou and Patrick 1976; An et al. 2001; Chung et al. 2001; Park et al. 2006), from root exudates (Pramanik et al. 2000; Seal et al. 2004), leaves (Singh et al. 1989; Chung et al. 2000), and bark of *Populus Canadensis* (Koopmann 2005). Salicylic acid induced several effects depending on the concentration applied. When exceeding a concentration of 0.5 mM, it caused a strong retardation of growth. *Arabidopsis thaliana* appeared bleached, presumably because salicylic acid induced an oxidative stress (Metraux et al. 1990; Rao et al. 1997; Rajjou et al. 2006). Salicylic acid can significantly affect mineral absorption in plants in the field (Harper and Balke 1981), stomatal movements and chlorophyll content (Manthe et al. 1992; Pancheva et al. 1996; Moharekar et al. 2003), and protein content and the rate of photosynthesis were reduced in barley plants by salicylic acid (Pancheva et al. 1996). In the range of 3 to 5 mM, salicylic acid completely inhibited maize embryo germination (Guan and Scandalios 1995). *R. formosanum*, *C. formosensis*, and *C. obtusa* var. *formosana* could grow on filter paper with 1.5 mM salicylic acid, but lettuce, *M. transmorrisonensis*, *P. taiwanensis*, and *B. formosana* did not.

Those species that could not grow or were inhibited on the bark may indeed be affected by salicylic acid. However, it was interesting to find that the two closely related species, namely, *C. formosensis* and *C. obtusa* var. *formosana*, could survive on paper with salicylic acid, but only *C. obtusa* var. *formosana* could grow on bark of *C. obtusa* var. *formosana*. Salicylic acid showed a significant inhibition of *B. pilosa* growth, but the extent of the inhibition was much less than that on the tree bark. Therefore, overall, there is not a very good correlation between effects of salicylic acid and effects of the tree bark when all species are compared. This suggests that salicylic acid may be only part of the story or may be important only for some species. The logs from which we collected bark for this study had fallen down between 0.5 and 2 yrs ago. Although outer layers of the bark from living *C. obstusa* var. *formosana* (ex. 1,000 yrs old) and downed logs are dead tissue and had already been flushed by rain numerous times, salicylic acid was still isolated. How such water-soluble allelochemicals persist in an ecosystem is an interesting question worth further experimentation.

In YYL forest, the canopy gaps created by *C. obtusa* var. *formosana*-downed logs would increase the level of light and provide new seedbeds where seedlings would not be

damaged by flooding from extremely high lake levels during typhoons and monsoons. Because the early growth of naturally regenerated *C. obtusa* var. *formosana* seedlings is slow, they often are suppressed by competing vegetation (Chiu et al. 1993). The bark seedbeds would provide safe sites for emerging seedlings and exclude competition from weeds and shrubs on the forest floor. The allelopathic effect of bark of the newly downed logs might act as a selective pressure that differentially affects various tree species. The two dominant species, *C. obtusa* var. *formosana* and *R. formosanum*, which can grow normally on the bark, could establish themselves on the downed logs and then occupy the gap, which would function as their regeneration niches. Further studies are needed to elucidate the source of the salicylic acid or additional allelochemicals or inhibitors. The interference factors might be due to logs of *C. obtusa* var. *formosana* themselves, or to lichens, fungi, or other microorganisms that distribute on the bark surface.

Acknowledgments The authors express their appreciation to the Forest Conservation and Management Administration, Veterans Affairs Commission, Executive Yuan, Taiwan, for permission to do this study in Yuan-yang Lake Nature Preserve and to Dr. T. H. Wang, Institute of Plant and Microbial Biology, Academia Sinica for laboratory assistance. This study was supported by Grants NSC-92-2313-B133-001 to Y. H. Kuo and M. W. Tseng.

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J Chem Ecol (2007) 33:1297
DOI 10.1007/s10886-007-9299-9

ERRATUM

Kairomones Extracted from Rice Yellow Stem Borer and their Influence on Egg Parasitization by *Trichogramma Japonicum* Ashmead

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Published online: 1 May 2007
© Springer Science + Business Media, LLC 2007

This paper, originally published in Online First and subsequently in Journal of Chemical Ecology 33:59-73 (2007), has been retracted. Critical data in this paper are not valid. This paper should not be referenced by authors writing on related topics.

The online version of the original article can be found at 10.1007/s10886-006-9203-z.

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Methods and Materials

Adult EAB were shipped under Animal and Plant Health Inspection Service (APHIS) permit from Brighton, MI, USA, to the Peoria, IL, USA, laboratory within a day of emergence from infested ash logs brought indoors in winter to induce EAB emergence. The beetles were kept segregated by sex at 27°C under a 16:8 (L/D) hour photoperiod and fed foliage of evergreen ash (*Fraxinus uhdei*). Volatiles were collected from both feeding males and females (five per collector) and from foliage alone (135, 119, and 10 total collections, respectively). Apparatus and general procedures were as described by Bartelt et al. (2006) for another beetle species. Volatiles were recovered from filters, foliage was renewed, and any dead beetles were replaced on a daily basis. The methods and equipment of Cossé and Bartelt (2000) were used for gas chromatography–electroantennographic detection (GC-EAD) analysis, except that excised antennae replaced whole-head preparations; 43 beetle-derived samples were analyzed. Equipment for GC-mass spectrometry (GC-MS), GC with flame ionization detector (GC-FID), and nuclear magnetic resonance spectroscopy (NMR) was as described previously (Bartelt et al. 2006). Compound quantitation was by the internal standard method (GC-FID, using heptadecane). Some collections were purified on small open columns of silica gel, eluting with increasing concentrations of diethyl ether in hexane. Purified beetle-derived material (silica gel, 10% ether in hexane) and synthetic lactone were hydrogenated over 10% Pd on carbon and were also derivatized with dimethyl disulfide (DMDS), procedures explained by Attygalle (1998). Chromatographic separations and reactions were monitored by GC-MS. (3*Z*)-dodecen-12-olide was synthesized, and the acquired ^{1}H and ^{13}C NMR spectra matched those reported (Boden et al. 1993).

Results and Discussion

One compound of apparent beetle origin (Fig. 1, arrow) was observed in volatile collections, in addition to plant-related compounds (Fig. 1, a and b). The beetle substance and some from the foliage were antennally active (Fig. 1, c and d). The indicated compound was consistently present in collections from females (129 out of 135, representing eight shipments of beetles), but it was also found in collections from males (52 out of 119) in smaller amounts, near the limit of detection. Mean initial emission rate from females was 10.7 ng/d per beetle (N=22 collections, SD=5.5, age 2–4 d after emergence), whereas the corresponding mean for males was 0.9 ng/d per beetle (N=20 collections, SD=0.6). Emission rates for both females and males decreased over time, being 26 and 19% of the initial values, respectively, for collections 6–7 d after emergence; this drop may simply reflect declining beetle health (typical mortality for shipments was 60% at 7 d after emergence), or it may be a natural pattern. The compound was never detected from foliage. No other antennally active compounds were recognized as likely coming from EAB.

The compound had the polarity of an ester on silica gel, eluting with 5–10% ether in hexane. The mass spectrum (EI, 70 eV) was not in the spectral library: [m/z (%)=196 (M^+, 8), 178 (7), 136 (19), 126 (8), 121 (13), 112 (27), 95 (35), 81 (66), 67 (92), 54 (100), 41 (73)]. Hydrogenation over Pd increased the molecular weight by 2, indicating the compound had one double bond [m/z (%)=198 (M^+, 2), 180 (5), 162 (7), 138 (19), 110 (23), 98 (61), 96 (46), 84 (47), 83 (51), 82 (38), 69 (53), 55 (100), 41 (71)]. This spectrum closely matched12-dodecanolide in the MS library. Therefore, dodecen-12-olides were explored in the literature, and (3*Z*)-dodecen-12-olide had a mass spectrum similar to that observed (Millar et al. 1985; Boden et al. 1993). Interestingly, this lactone is the major component of the male-produced pheromone of the flat grain beetle *Cryptolestes pusillus*

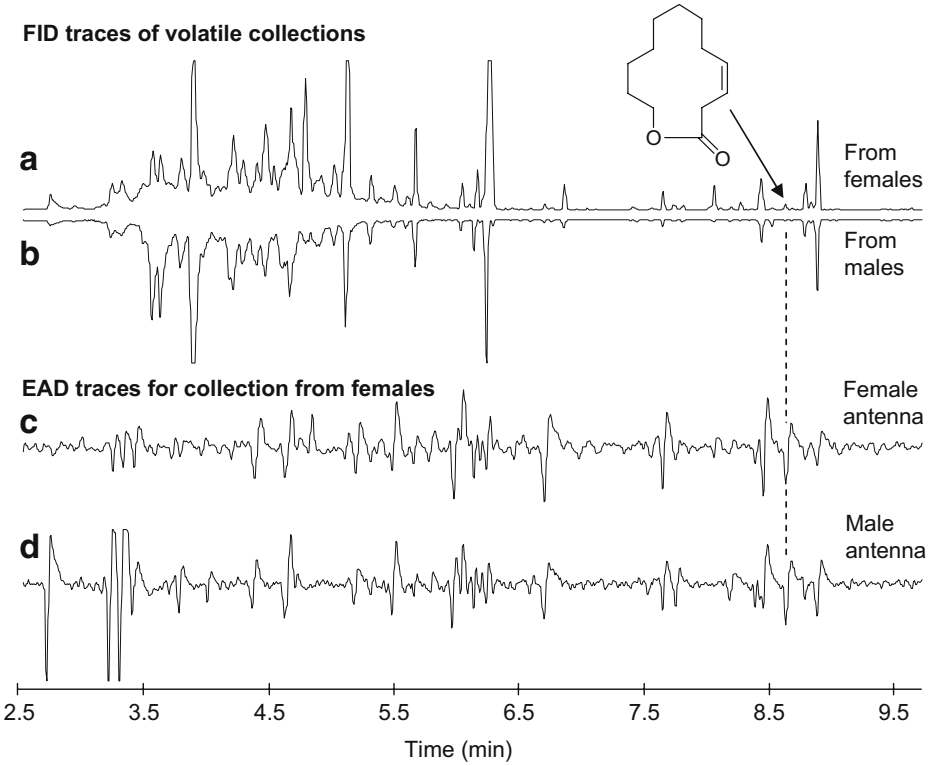


Fig. 1 GC-FID comparison of volatiles collected from female (**a**) and male (**b**) *A. planipennis* feeding on ash foliage and GC-EAD traces of female (**c**) and male (**d**) antennae to a female collection. Structure of (3*Z*)-dodecen-12-olide is shown

(Schönherr) (Coleoptera: Cucujidae) (Millar et al. 1985). It was synthesized as a standard for comparison.

Synthetic (3*Z*)-dodecen-12-olide was identical to the beetle compound by GC retention, mass spectrum, hydrogenation product, and polarity on silica. The trace of *E* isomer in the synthetic lactone (2%) eluted before the beetle compound. The DMDS derivatives of the natural and synthetic lactones gave identical mass spectra [m/z (%)=290 (M^+, 35), 243 (15), 227 (18), 122 (100)]. Mass spectra of lactone DMDS adducts are more complex than open-chain olefins, but direct comparisons would still reveal differences in double-bond position. Together, these data established that the beetle compound was (3*Z*)-dodecen-12-olide (Fig. 1). The synthetic lactone was active by GC-EAD.

Our GC-EAD results were consistent with patterns of field attraction reported for another buprestid, *Agrilus bilineatus* (Weber), in that both males and females responded positively to females (Dunn and Potter 1988); attraction to male *A. bilineatus* was not investigated, and no chemical details were reported. If the lactone has a pheromonal function in EAB, it is likely not a typical sex pheromone because it was detected from both sexes and because the greatest emission (2–4 d after emergence) occurred before the beetles were expected to be sexually mature (e.g., 7 d after emergence, Bauer et al. 2003). An aggregation function is possible, but additional mechanisms such as visual cues are undoubtedly involved in mate finding (Lelito et al., unpublished). Further research is needed to characterize any behavioral activity toward

the EAB lactone, but the urgent need to improve monitoring makes this a high priority. Such studies are planned.

Acknowledgment We thank Karl Vermillion of NCAUR for acquiring the NMR spectra.

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J Chem Ecol (2007) 33:1303–1315
DOI 10.1007/s10886-007-9320-3

Electrophysiological and Behavioral Responses of *Ips duplicatus* to Aggregation Pheromone in Inner Mongolia, China: Amitinol as a Potential Pheromone Component

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Received: 24 April 2007 / Revised: 15 May 2007 / Accepted: 17 May 2007 /
Published online: 9 June 2007
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Abstract Volatiles from *Ips duplicatus* male hindgut extracts and aeration samples of spruce logs colonized by the virgin males were analyzed by coupled gas chromatography–mass spectrometry (GC-MS), coupled gas chromatography–electroantennographic detection (GC-EAD), and field trapping bioassays in Inner Mongolia, China. GC-MS analyses showed that males from nuptial chambers (for reproduction) produced hindgut volatile profiles similar to those of maturation feeding males from the nonreproductive galleries. The known aggregation pheromone components, ipsdienol (Id) and *E*-myrcenol (EM) are the major constituents of this blend, followed by several minor bark beetle-related compounds: 2-methyl-3-buten-2-ol, ipsenol, *cis*-verbenol, verbenone, 2-phenylethanol, myrtenol, and *trans*-myrtanol. The enantiomeric composition of the Id from the male hindgut extracts was determined to be a racemic mixture (50/50) by enantioselective GC. GC-EAD experiments indicated that *I. duplicatus* antennae from both sexes responded strongly not only to the known aggregation pheromone components, Id and EM, but also to the other minor conspecific-produced compounds. The strongest antennal response was elicited by the major pheromone component, Id, which was ca. 2–3 times higher than that elicited by the second component, EM. Electroantennogram dose-responses indicated that antennal response threshold to Id was

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approximately 100 times lower than that of EM. No synergistic effects between these occurred at peripheral level. A strong and repeatable EAD response was found to a trace compound (flame ionization detection undetectable) from both hindgut and aeration samples. The compound was identified as amitinol (At) based on the retention time and a further EAD analysis of a synthetic mixture. In the field trapping experiments, At increased the attraction of *I. duplicatus* to the traps baited with a binary blend of EM/Id (1:1) in a synergistic manner when tested at a low release rate (1:1:0.1) but had no effect on catches at the high release rate (1:1:1). This result suggests that At might be a part of the Chinese *I. duplicatus* aggregation pheromone system. The production ratios of EM/Id from the Inner Mongolian population (EM/Id≈1:2.0–4) were twice as high as those reported from Europe (EM/Id≈1:9), corresponding well with the differences in the optimal response ratios.

Keywords Coleoptera · Scolytidae · Attractant · GC-EAD · GC-MS · Ipsdienol · *E*-myrcenol · Amitinol · Ipsenol · Enantiomeric composition

Introduction

The double-spined spruce bark engraver, *Ips duplicatus* (Sahlb.), is a spruce (*Picea* spp.) pest insect in Europe and North-East Asia (Lekander et al. 1977; Pfeffer 1995; Zhang et al. 2001). Recent outbreaks in Czech Republic (Knizek and Zahradnik 1996) and continuous outbreaks since the 1950s in Inner Mongolia, China (Zhang et al. 1995; Liu et al. 1998), as well as its status as a vector of phytopathogenic fungi (Krokene and Solheim 1996), pose a serious threat to the spruce forests in these regions.

The aggregation pheromone system of *I. duplicatus* has been intensively studied in Europe, where it consists of two components, racemic ipsdienol (Id) and *E*-myrcenol (EM) (Bakke 1975; Schlyter et al. 1987, 1992; Byers et al. 1990). West palearctic (Norway/Sweden) *I. duplicatus* contain a blend of EM/Id (ca. 1:9), and a synthetic blend of these *de novo* produced components (Ivarsson et al. 1993) is highly competitive to natural pheromone emitted by unpaired males in spruce logs (Schlyter et al. 1992). A recent study comparing the optimal EM/Id proportions for attraction across four populations (Norway, Czech Republic, Great Xingan Mountains, and Inner Mongolia, China) revealed that the Inner Mongolia population has a significantly different optimal response ratio of EM/Id from other populations (Schlyter et al. 2001a). This finding raises questions concerning the production ratio of the two aggregation pheromone components (Id and EM), the enantiomeric composition of Id in the Inner Mongolia population and its potential genetic divergence from the European populations (Lakatos et al., unpublished). Furthermore, a fraction of the *I. duplicatus* population in Inner Mongolia require maturation feeding on wind-thrown or weakened spruce trees in early summer for 1–2 weeks before initiating the normal attacks on standing trees for reproduction (Wang 1992; Zhang et al. 1995). Such a maturation feeding on logs or felled trees demonstrates a strong aggregation pattern, with 20–50 adults (ca. 1:1 of sex ratio) feeding in a single entry "communal" gallery.

In the current study, we tested the following four hypotheses on the Inner Mongolian population of *I. duplicatus*: H_1, "communal maturation feeding" males differ from normal colonizers of fresh host trees in their semiochemical productions; H_2, there is a correspondence between the production and response of the two main pheromone components (and/or their ratios) among the different geographical populations (such as West palearctic and Inner Mongolia); H_3, the enantiomeric composition of the major

pheromone component Id differs between the Chinese and the European populations; and H_4, additional pheromone components may exist that act synergistically with the two main components. These hypotheses were tested by using gas chromatography–mass spectrometry (GC-MS), gas chromatography–electroantennographic detection (GC-EAD), and field trapping bioassays.

Materials and Methods

Field Site

All beetle collections and field trapping experiments were carried out in the National Baiyinaobao Spruce Natural Reserve [*Picea mongolica* (Wu, H.-Q.) Xu, formally *Picea koraiensis* Nakai or *Picea meyeri* or *P. meyeri* var. *mongolica* Wu, as discussed by Xu et al. (1998)] at Keshiketeng Qi (43° 30′–36′ N, 117° 06′–16′ E), Inner Mongolia, China, which is situated in the grassland with a thick layer of sandy soil at the east edge of Hunshandake desert. Since the 1950s, this isolated natural spruce forest has been severely infested by sawflies and *I. duplicatus* (Zhang et al. 1995, 2001; Liu et al. 1998; Schlyter et al. 2001b).

Hindgut Extraction and Headspace Sampling

Field Sampling Ips duplicatus adults were collected at the field site in early June 1994 during natural attacks on *P. mongolica*. Infested spruce trees were cut, and logs exhibiting either beetle maturation feeding in communal chambers or reproduction in nuptial chambers (with the status of one male and 1–2 females per family and no eggs) were transported to the local laboratory. Live beetles caught in pheromone traps were also collected at this time. Hindguts were rapidly dissected from the beetles, extracted in 300 μl of pentane, and pooled separately according to sex and attacking phases (Zhang et al. 2000). The extracts (9–22 guts/sample) were kept at −20°C prior to GC-MS analysis in Sweden.

Laboratory Collection A laboratory culture of live beetles originally collected from the same site as for the field collection/trapping in Inner Mongolia, China, was successfully maintained on Norway spruce (*Picea abies*) logs from 1999 to 2000 at the Swedish University of Agricultural Sciences, Alnarp, in Sweden. Twenty to 30 males, emerged from generation 3 (March 2000), were allowed to colonize 10-cm-diameter spruce bolts with predrilled holes (25°C, 70% RH, 20:4 L/D). After feeding for 24 hr, the bolts with male beetles were placed into polyester bags (Meny Toppits, Terinex, Bedford, England), each equipped with a charcoal filter tube. Headspace volatiles of the male-infested bolts were collected on 30-mg Porapak Q (50–80 mesh, Supelco, Bellefonte, PA, USA) in a 3-mm ID Teflon tube via a portable aeration system as described by Zhang et al. (1999) for 1.5 hr. The Porapak Q collections were each rinsed in 300 μl of diethyl ether. Immediately following headspace collection, the males were excised from the nuptial chambers and hindguts were extracted in 300 μl of hexane. Headspace from an uninfested spruce bolt of similar size taken from the same tree was collected in parallel to serve as a control. Both hindgut extracts and aeration samples were kept at −20°C until GC-EAD and GC-MS analysis. For enantiomeric analysis of Id, a new batch of wild beetles was collected from the same location in Inner Mongolia in October 2006, and beetles were allowed to colonize spruce logs for 24 hr in the laboratory before hindgut extraction with hexane.

GC-MS Analysis

Hindgut extracts from field-collected beetles (in 1994) were analyzed on a combined Hewlett-Packard (HP) 5890 series II gas chromatograph (GC) and an HP 5972 mass selective detector (MSD). Laboratory aeration and hindgut samples (in 2000) were analyzed on a newer model of GC-MSD (HP 6890 GC–HP 5973 MSD). Both GCs were equipped with a 25 m×0.25 mm fused silica column coated with 0.25 μm OV-351 (Ohio Valley Speciality Chemical, Marietta, OH, USA). The GC oven temperature was programmed at 50°C for 2 min, with a rise to 200°C at a rate of 8°C/min, and held at 200°C for 5 min. To clear the column from contaminants, the temperature was then raised at 20°C/min to 250°C and held for 5 min. Injector and transfer line temperatures were maintained at 200 and 225°C, respectively. Helium was used as carrier gas, and the flow was electronically controlled to be a constant 30 cm/sec. Volatiles were identified by comparisons of the retention times and mass spectra with those from the available authentic synthetic compounds, along with those from the computerized data library, NBS75K, and with a custom produced library (KE 1995, Göteborg).

Enantiomeric Composition of Id

A new extract of male hindguts (N=19 in 20 μl of hexane; after 24 hr feeding in spruce log) was made from the freshly collected beetles (2006; see above). The extract (1 μl) was injected splitless into a Varian CP-3800 GC equipped with a fused silica column coated with an optically active stationary phase (Rt-bDEXm™; 30 m×0.25 mm×0.25 μm; Restek, Bellefonte, PA, USA). Helium was used as the carrier gas and the injector/detector temperatures were all 230°C. The column temperature was 80°C for 1 min and rose to 200°C at 2°C/min. To confirm the retention times of enantiomers, a synthetic mixture of ipsenol (Ie), amitinol (At), and Id (100 ng/μl each) was subsequently injected.

GC-EAD and Electroantennogram Responses

Male hindgut extracts or aeration samples (2–3 μl) and synthetic mixture (1–2 μl; consisting of Ie, Id, At, and EM; 30 ng/μl each) were splitlessly injected into an HP-6890 GC equipped with an HP-INNOWAX column (30 m×0.25 mm×0.25 μm). A 1:1 effluent splitter allowed simultaneous flame ionization detection (FID) and EAD of the separated volatile compounds. Hydrogen was used as the carrier gas. The column temperature was 40°C for the first 2 min, rising to 200°C at 10°C/min, and held for 2 min. The outlet for the EAD was inserted into a humidified air-stream (0.5 m/sec) over an *I. duplicatus* antennal preparation. A glass capillary indifferent electrode, filled with Beadle–Ephrussi Ringer solution and grounded via a silver wire, was inserted into the severed beetle's head with the antennae. A similar recording electrode, connected to a high-impedance DC amplifier with automatic baseline drift compensation, was placed in contact with the distal end of the antennal club. The resulting signals were stored on a PC equipped with an intelligent data acquisition controller card and analyzed with the program EAD (version 2.3, Syntech, Hilversum, the Netherlands). For each GC-EAD analysis, *I. duplicatus* antennae from three females and two males were tested.

Electroantennogram (EAG) dose-responses of *I. duplicatus* to synthetic samples of two known pheromone components, Id and EM, were recorded under similar conditions. Stimuli were prepared by applying Id and EM separately and together at a 1:1 ratio (10^{-4} to 10^{1} μg/μl) in 2 μl of hexane on a piece of filter paper (7×15 mm) in a Pasteur pipette. The same total loading amounts on the filter paper were used for the blends as for the individual compound treatments. Compounds were tested at five concentrations from the lowest to the

highest. "Blank control" stimuli (2 μl of hexane solvent per filter paper) were interspersed between different compounds tested. Each stimulus was followed by a period of at least 60 sec of filtered humid air passing over the antenna to ensure recovery of antennal receptors.

Field Trapping

Eight black flight barrier traps (Theyson, Germany) were set up in mid-June, 2001, in a small glade surrounded by a *P. mongolica* stand with dead trees that were killed by *I. duplicatus* the previous year. Traps were arranged in an arc to ensure a similar distance (ca. 20–25 m) from the insect source (the dead trees) for each trap. The distance between traps was ca. 8 m. Two previously known pheromone components, Id and EM (Byers et al. 1990; Schlyter et al. 1992), along with a novel EAD-active compound, At, were tested by using a factorial design, i.e., individually and in all possible combinations. A similar test with a reduced number of treatments was repeated in the same site in early June 2002. Traps together with dispensers were rotated after each replicate (when ≥80–100 beetles were caught in the best trap) in a randomized Latin-square design. All chemicals, source, release devices, and release rates are listed in Table 1.

Statistical Analysis

Trap catch data were transformed by $\log(x+1)$ to fit the assumption of homogeneity of variance for ANOVA. Means were compared by ANOVA followed by the Ryan–Einot–Gabriel–Welsh multiple Q test (SPSS 8.0 for Windows) at $\alpha=0.05$. The sex ratios for the treatments within each experiment were compared with 95% binomial confidence intervals (Newcombe 1998, 2006).

Results

GC-MS Analysis of Hindgut Extracts

Male and female *Ips* beetles are in a nonfeeding phase during dispersal flights. Dispersing beetles of both sexes collected from pheromone traps contained no bark beetle pheromone compounds. Males in the communal maturation feeding chambers produced both Id and EM as major components, with the ratio of EM/Id at ca. 1:4 (Table 2). In addition, several other compounds frequently found in *Ips* beetles, 2-methyl-3-buten-2-ol, Ie, *cis*-verbenol (cV), verbenone (Vn), 2-phenylethanol (PE), myrtenol, and *trans*-myrtanol, were also identified in

Table 1 Chemicals, release rates, and dispensers used in field-trapping experiments

Chemical	Source	Purity (%)	Release rate (mg/24 hr)[a]	Loading (mg)	Dispensers[b]
(±)-Id	ICN Biomedicals, USA	95	0.3	20	One open 2-ml glass vial
(*E*)-myrcenol (EM)	SciTech, Czech Republic	95.2	0.1	20	One open 2-ml glass vial
At	W. Francke, Germany	98	0.85	20	One open 2-ml glass vial
	W. Francke, Germany	98	0.06	2	Mixed with 20 mg ID in one open 2-ml glass vial

[a] Measured in a mini wind-tunnel at 20–21°C and 0.7 m/sec for 1 week

[b] Glass vial with 5-mm-diameter opening, 32-mm height

Table 2 Hindgut and head-space volatiles of *I. duplicatus* from different attacking phases (Inner Mongolia, China), identified by GC-MS

Chemicals	Hindgut extracts							Headspace sampling (20 M feeding for 24 hr)[b]	EAD activity
	Traps		Maturation feeding		Reproduction feeding				
	M (*N*=22)[a]	F (*N*=22)	M (*N*=22)	F (*N*=20)	M (field) (*N*=9)	F (field) (*N*=13)	M (lab) (*N*=20–30)		
2-Methyl-3-buten-2-ol					+		++	+	
1-Hexanol							Trace	Trace	*
Nonanal		+	+	+		Trace	+	+	
Benzaldehyde							+	+	
Ie			Trace		Trace		Trace	Trace	*
Bornyl acetate							+	+	
At							?	?	*
Unidentifed (Bp=59>m/z 102)		+	Trace	+++		Trace			
Unidentified (Bp=91>m/z 120)		+		+	+				
cV			+		++		++	++	*
Id			+++	Trace	+++		+++	+++	*
Trans-Verbenol							Trace	+	*
Unidentified (Bp=120> m/z 152, 92)			Trace	+	+				
Vn							Trace	+	*
Myrtenol			Trace		+		+	+	
Geraniol							+	+	
Trans-Myrtanol			Trace		Trace		Trace	Trace	
2-Phenylethanol (PE)			+		Trace				
EM			++		++		+++	+++	*
Perilla alcohol							Trace	Trace	
Ratio of EM/Id			1:4		1:6		1:2.0–2.5	1:4–6	

Trace: <1% of total volatiles; +: <5% of total volatiles; ++: minor component, >5% of total volatiles; +++: major component, >25% of total volatiles; ?: Detected by EAD, but not by FID; *: antennally active

[a] Number of hindguts in the extracts

[b] Only the beetle-produced volatiles are considered

the males in trace or minor amounts. No bark beetle-specific compounds were detected from the hindguts of maturation feeding females, except a trace amount of Id. Males from nuptial chambers displayed similar hindgut volatile profiles, both qualitatively and quantitatively, to the maturation feeding males (Table 2). The ratios of EM/Id were estimated as ca. 1:4 to 1:6. Females from the nuptial chambers contained no bark beetle pheromone compounds at detectable level.

Male hindgut and headspace extracts from the laboratory cultures showed a similar GC pattern of male-produced volatiles to the field-collected samples, with both Id and EM as the major components (Table 2; Figs. 1b and 2a). However, the ratio of EM/Id appears to be higher in hindgut extracts (1:2 to 1:2.5) than that in the aeration samples (1:4 to 1:6). In addition to the well-known bark beetle compounds, trace amounts of a green leaf volatile (GLV), 1-hexanol, were also detected from both samples. No Id and EM were identified from the headspace samples of spruce bolts alone, but trace amount of 1-hexanol was detected in addition to large amounts of monoterpenes (Fig. 1a).

Enantiomeric Composition of Id

Enantioselective analysis indicated that males of *I. duplicatus* produced a racemic mixture (50/50) of the major component, Id (Fig. 3a), which was confirmed by the synthetic mixture (Fig. 3b).

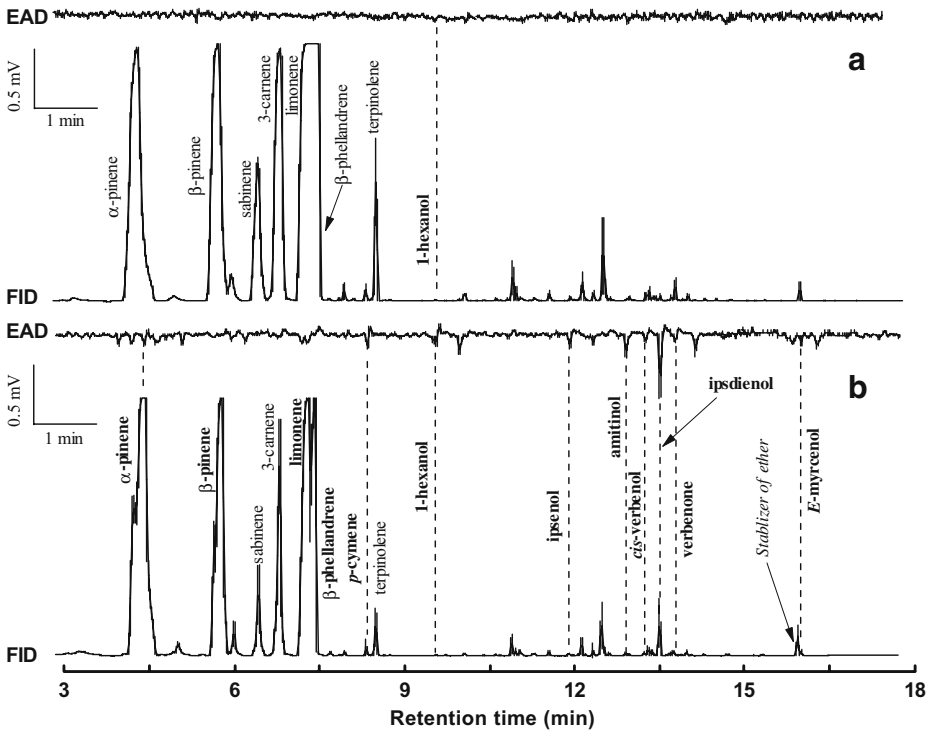


Fig. 1 Simultaneously recorded FID and EAD responses of *I. duplicatus* antennae to aeration samples of **a** a spruce log and **b** a spruce log colonized by 25 males for 24 hr in the lab. EAD-active compounds are labeled in *bold*

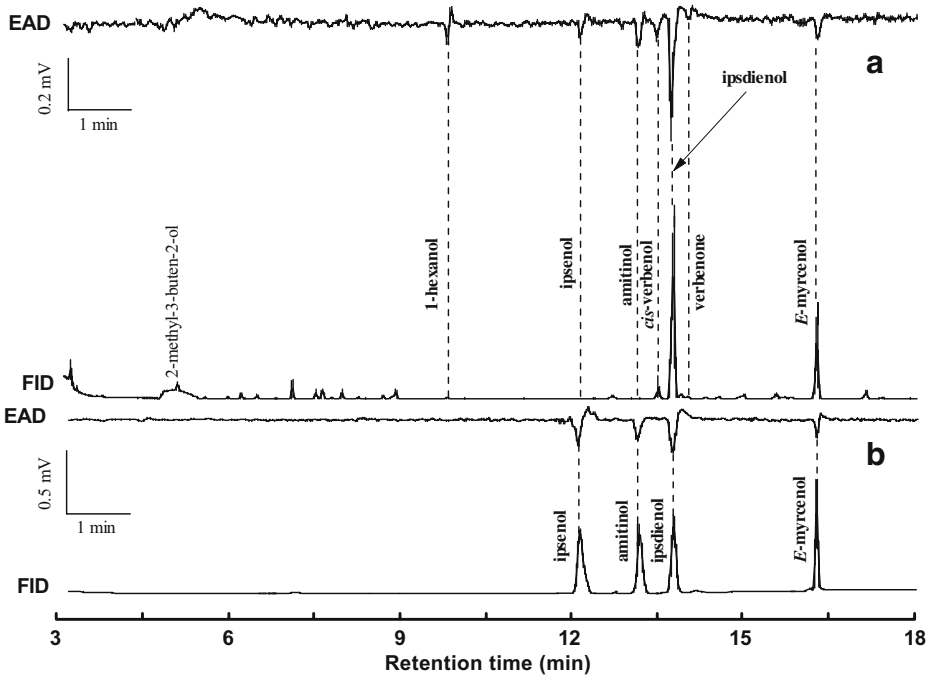


Fig. 2 Simultaneously recorded FID and EAD responses of *I. duplicatus* antennae to hindgut extracts of the males fed in the spruce logs for 24 hr (**a**) and to a synthetic mixture of selected bark beetle pheromone compounds (**b**). EAD-active compounds are labeled in *bold*

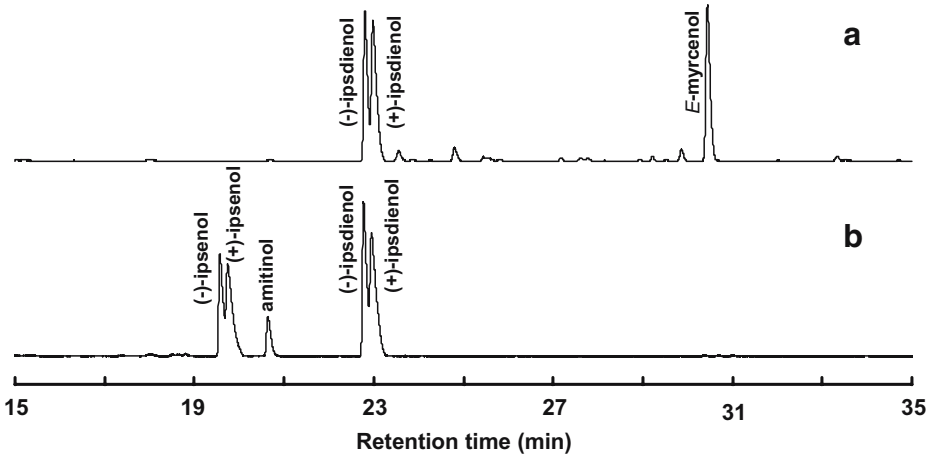


Fig. 3 Enantioselective GC analysis of Id from **a** an *I. duplicatus* hindgut extract of 19 unmated males making nuptial chambers in Norway spruce (*Picea abies*) log for 24 hr and **b** a synthetic mixture of racemic Ie, At, and racemic Id. The elution orders of (+) or (−) enantiomers of Ie and Id were determined according to the results reported in Macias-Samano et al. (1997) and Savoie et al. (1998), of which similar type of column (with the same stationary phase as ours) was used. For Ie and Id, (−)-enantiomers should elute before its corresponding (+)-enantiomers

GC-EAD Recordings and EAG Dose Responses

Coupled GC-EAD analyses on headspace extracts of spruce bolts alone revealed reproducible responses to a trace amount of 1-hexanol and occasionally weak responses to a few monoterpenes such as α-pinene, β-pinene, limonene, β-phellandrene, and *p*-cymene (Fig. 1a). In addition to the weak monoterpene and reproducible 1-hexanol responses, strong EAD responses were found to six additional compounds from both headspace samples of male-infested bolts (Fig. 1b) and male hindgut extracts (Fig. 2a). The strongest EAD responses were recorded to Id, followed by a FID undetected compound, then EM, cV, Ie, and Vn (Fig. 1b, Fig. 2a). Based on the retention time and a further EAD analysis of a synthetic mixture (Fig. 2b), this GC-FID undetectable, antennally active compound was identified as At. No antennal activity to 2-methyl-3-buten-2-ol was found, although the amount produced was comparable to the two major hindgut components, Id and EM (Fig. 2a). No differences in antennal responses between males and females were found.

EAG dose-responses were tested against solvent control to compare the relative sensitivity of *I. duplicatus* antennae to the two major pheromone components, Id and EM, and their potential synergistic effect at a peripheral level. For the three treatments tested, there was a significant effect of dosage on the EAG response (Fig. 4). However, the response threshold to Id (0.1–1 ng/μl) was approximately 100 times lower than that of EM. The dose-response curve for the Id/EM blend was identical to Id alone, which indicated no synergistic effect between these two pheromone components at peripheral level.

Field Trapping

In a field trapping experiment carried out in 2001, a total of 3,848 beetles were caught during 16 replicates. Individual compounds, Id, EM, At, and two binary blends, Id + At and EM + At, caught zero or less than 35 beetles, which were not significantly different from zero. However, the combination of Id and EM was attractive with its total catch of over 1,100 beetles (ca. 35–60 times higher than the individual Id or EM treatments). Addition of At at a high release rate to this mixture (EM/Id/At=1:1:1) did not increase trap catches, whereas a low rate of release of At (EM/Id/At=1:1:0.1) significantly enhanced the numbers of beetles caught by 47% (Fig. 5a). A similar enhancement in trap catches by adding At at a

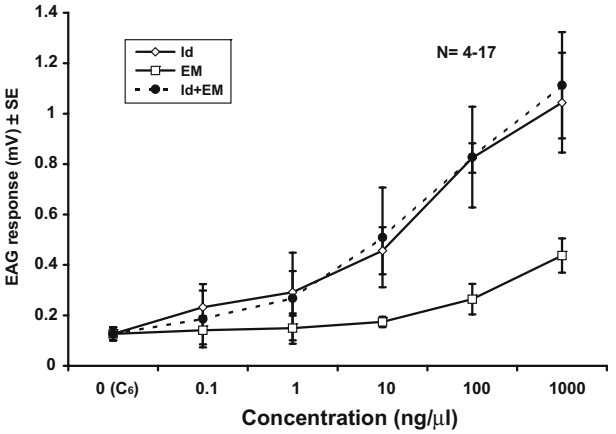


Fig. 4 Responses of *I. duplicatus* antennae (EAG) to various doses of Id, EM, and their combination (Id + EM). Doses are given as nanograms applied to filter paper in the stimulus pipette in hexane solvent

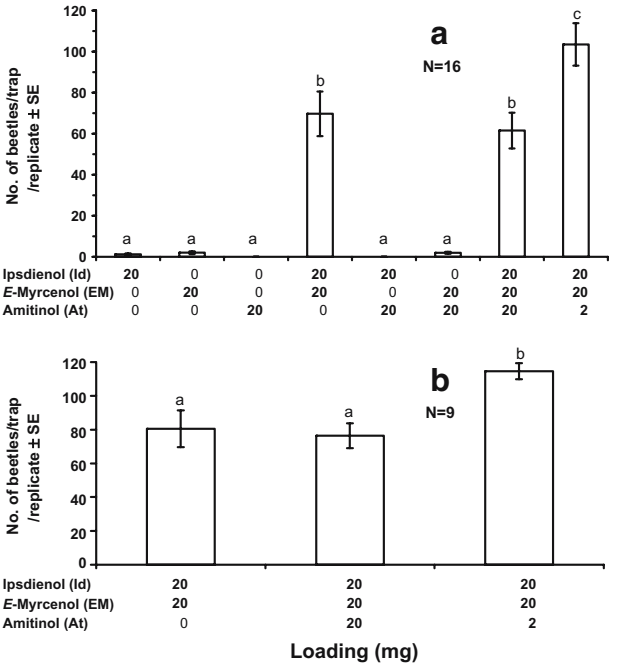


Fig. 5 Captures of *I. duplicatus* in traps baited with **a** the individual pheromone components, Id, EM, and At with all their binary and ternary combinations, June 24–27, 2001, Baiyinaobao, Inner Mongolia, China, and **b** in traps baited with a binary blend (Id/EM) and ternary mixtures with two levels of At release; June 1–3, 2002, Baiyinaobao, Inner Mongolia, China. Bars with the same letter are not significantly different ($P>0.05$) by Ryan–Einot–Gabriel–Welsh multiple Q test after ANOVA on log ($x+1$) transformed data

low rate of release (EM/Id/At=1:1:0.1) was also found in the 2002 field trapping experiment (Fig. 5b). There were no significant differences in sex ratios among different treatments for the two experiments; while the 2002 experiment conducted earlier in the flight period had a much higher "percent males" for comparable baits (Table 3).

Table 3 Proportion of *I. duplicatus* males captured

Test/treatment (mg)	% of Males	95% CI[a]	Total catch
Experiment 1: June 24–27, 2001			
Id (20)	21.1	8.5–43.3	19
EM (20)	25.0	13.3–42.1	32
At (20)	–	–	0
Id + EM (20+20)	11.2	7.3–16.9	1,125[b]
Id + At (20+20)	–	–	0
EM + At (20+20)	25.8	13.7–43.3	31
Id + EM + At (20+20+20)	16.6	11.7–22.9	985[b]
EM + Id + At [20+(20+2)]	10.6	6.9–15.9	1,656[b]
Experiment 2: June 2–4, 2002			
Id + EM (20+20)	27.59	20.27–36.34	724[b]
Id + EM + At (20+20+20)	26.09	18.2–35.89	688[b]
EM + Id + At [20+(20+2)]	29.52	21.65–38.85	1,032[b]

[a] 95% binomial confidence intervals (Newcombe 1998, 2006)

[b] Subsamplings of 100–180 beetles were taken for determination of sex ratios

Discussion

This is the first chemical analysis of the aggregation pheromone system of an Asian population of *I. duplicatus*. Coupled GC-MS results demonstrate that overwintered *I. duplicatus* from Inner Mongolia produce no pheromone components during the early summer prefeeding dispersal flight. Males from the maturation feeding galleries had a hindgut volatile profile similar to the normal reproductive feeding males, with Id and EM as the major pheromone components (Table 2). Other compounds frequently found in *Ips* beetles, such as Ie, cV, PE, myrtenol, and *trans*-myrtanol, were also detected, but mainly in minor or trace amounts. These results reject our first hypothesis concerning the production of different semiochemicals between maturation feeding males and normal reproductive feeding males. The mechanism by which dispersing individuals separate reproductive feeding males from ones under maturation feeding, therefore, remains unknown, because no differences in hindgut volatile profiles were detected. In comparison with European populations, there was no qualitative difference of male hindgut volatiles of normal colonizers; however, the production ratios of EM/Id from the Inner Mongolian population were twice as high as those of the European ones (EM/Id≈1:9) (Schlyter et al. 1992). This corresponds well with the differences in the optimal response ratios from trapping tests (Schlyter et al. 2001a). Thus, this Chinese population not only produces but also prefers a higher proportion of EM in the binary blends than do European populations. Such correspondence supports our second hypothesis on pheromone productions and responses. Enantioselctive GC analysis indicated that males of the Chinese *I. duplicatus* produced their major pheromone component Id in a racemic form, similar to that of the European populations (Schlyter et al. 1992), which leads to the rejection of our third hypothesis on a geographical variation in enantiomeric composition of Id.

Monoterpenes, major components from the headspace samples of the uninfested and the infested spruce logs, elicited weak or no EAD activities at the dosages tested. Interestingly, a trace amount of 1-hexanol was detected from both aeration samples and male hindgut extracts and showed a reproducible EAD-response. 1-Hexanol has been reported previously in the hindguts of *Pityogenes chalcographus* (Francke et al. 1977) and *I. subelongatus* (Zhang et al. 2007) males. This compound is a common GLV and has been considered as one of major EAD-active nonhost volatiles for many conifer-infesting bark beetles, including the *I. duplicatus* (Zhang et al. 1999, 2001). 1-Hexanol alone was inactive as an attraction-inhibitor (at 4 mg/d) but was significantly inhibitory when combined with two other GLV alcohols (Zhang et al. 2001). The function of 1-hexanol (at the low level) from the spruce logs or the hindgut extracts on the host selection behavior of *I. duplicatus* remains unknown.

Our GC-EAD analyses revealed that *I. duplicatus* antennae from both sexes responded strongly, not only to the known aggregation pheromone components, Id and EM, but also to other minor conspecific-produced compounds. EAG dose responses indicated that the antennal response threshold to Id was approximately 100 times lower than that of EM, and no synergistic effects between these two known pheromone components were identified at peripheral level. Strong EAD responses to Ie, cV, Vn, and a FID-undetected compound from both hindgut and aeration samples were also found. Ie, cV, and Vn were also identified from the European populations and were inhibitory to the pheromone attraction (Schlyter et al. 1992). The EAD-active, FID-undetected compound was identified as At and is a pheromone component first reported from *I. aminitus* (Eichhoff) (Francke et al. 1980) and later from *I. tridens* Mannerheim (Poland and Borden 1997). Similarly, At was not detected by GC-FID/MSD from *I. subelongatus* male hindgut extracts, but it was identified

with the aid of GC-EAD technique (Zhang et al. 2007). The present field trapping tests in Inner Mongolia showed that individual Id, EM, and At were inactive, but the binary blend of Id/EM (1:1) was attractive, while addition of a small amount of At to the blend significantly increased the trap catches. Therefore, At might be a component of the aggregation pheromone system of the Chinese *I. duplicatus*, which appears to support our fourth hypothesis that additional pheromone components may act synergistically with the two main components. Surprisingly, the addition of At (<1% of EM and Id) at the high release rate, similar to that of major pheromone components, had little negative effect on trap catches. The EAD detection of At from the hindgut extracts and aeration samples could result from actual production of the compound by the beetles or through acid rearrangement of Id during collection, extraction, storage, or analysis of the samples (Francke et al. 1980). No GC-EAD work has yet been done on the European populations of *I. duplicatus*. Thus, it will be interesting to see if European populations produce and respond in a similar fashion to a trace amount of At. Whether or not At is naturally produced or is a result of rearrangement during the natural production of Id, the synergistic effect (at a low release rate) on attraction to the Id/EM blend may further improve the trap performance for both monitoring and mass-trapping programs against this pest insect (Schlyter et al. 2001b).

It should be noted that our target population in the current study is from an isolated spruce nature reserve in the middle of a desert grass land in the eastern Palearctic (Zhang et al. 2001), which is isolated from other populations of *I. duplicatus* and *Picea*. Therefore, the evolutionary pressure of *I. duplicatus* population in Baiyinaobao, Inner Mongolia, together with its host tree, *P. mongolica*, might have been radically different from populations of the contiguous distribution in the western Palearctic (Europe). A recent study on the genetic structures as assessed by analysis of mitochondrial DNA sequence of *I. duplicatus* populations from Europe and Inner Mongolia, China (the same population as in the current study), revealed a 0.8% sequence divergence between the Asian and the European populations (Lakatos et al., unpublished). This genetic divergence may explain, in part, the differences in pheromone production and behavioral responses between the European populations and the isolated Chinese population.

Acknowledgements We thank Dr. F.-M. Yan, Beijing University, China, for his careful shipment of the live beetles; Dr. S. R. Hill for helpful comments on the manuscript; and Ms. E. Marling for help in the preparation of hindgut extracts for enantioselective analysis. The permission to "thin" a spruce stand at Björnstorp Estate for bark beetle breeding is highly appreciated. This study was supported by grants from SJFR (No. 23.0521/96; No. 24.0293/98) and FORMAS (230-2005-1778) and an EU-INCO project (CT 98-0151).

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Keywords *Dendrolimus houi* · Sex pheromone · Solid phase microextraction · (5*E*,7*Z*)-5,7-dodecadien-1-ol · (5*E*,7*Z*)-5,7-dodecadien-1-yl acetate · (5*E*,7*Z*)-5,7-dodecadienal

Introduction

The Yunnan pine caterpillar moth *Dendrolimus houi* Lajonquière (Lepidoptera: Lasiocampidae) is an economically important defoliator of coniferous forests in southwestern China, especially in Yunnan Province. The moth's preferred host is *Pinus yunnanensis* Franch., but it also feeds on other *Pinus* species, such as *P. langhianensis* Chev., *P. armandii* Franch., *P. massoniana* Lamb, and *P. densata* Mast., as well as *Platycladus orientalis* (L.) Franco, *Cupressus funebris* Endl., *Keteleeria fortunei* (Murr.) Carr., and *Cryptomeria fortunei* Hooibrenk ex Otto et Dietr. (Hou 1987; Chen 1990; Yin et al. 2002; Guo et al. 2003). In most counties of Simao prefecture, *D. houi* has two generations and overwinters as larvae. Adult flights usually occur in June/July and October/November. Most damage is caused by 4th–7th instars in March–April and in August. During outbreak years, *D. houi* can kill thousands to millions of hectares of forests (Chai et al. 2002). Outbreaks of this herbivore across southwestern China have stimulated extensive application of synthetic pyrethroids or naturally derived insecticidal compounds, which has resulted in severe negative effects on biodiversity and natural enemies of herbivores in the ecosystem. To control *D. houi* effectively, monitoring of its population outbreaks has been initiated (Chai et al. 2002; Zhao et al. 2003; Guo et al. 2003). Identification of the sex pheromone of *D. houi* was undertaken in the hope that the synthetic pheromone would be of value for population monitoring and for control by mating disruption (Kong et al. 2004).

There are five species in the genus *Dendrolimus* for which sex pheromones or attractants have been identified (Chen et al. 1980; Ando et al. 1982; Meng and Wang 1983; Priesner et al. 1984; Kovalev et al. 1993; Zhao et al. 1993; Klun et al. 2000; Kong et al. 2001, 2003, 2005; Khrimian et al. 2002; Zhao et al. 2004). The sex pheromones share a common chemical theme of being C_{12} (*Z*,*E*)-5,7-dienes with alcohol, acetate, propionate, or aldehyde functional groups.

The sex pheromone communication system of *D. houi* has received considerable attention because of its economic importance. However, until now its sex pheromone had not been elucidated. Recently, preliminary analyses of extracts of the sex pheromone glands of *D. houi* indicated the presence of (5*E*,7*Z*)-5,7-dodecadien-1-ol (*E*5,*Z*7-12:OH) and its corresponding acetate and aldehyde, and the conjugated double-bond position was fixed at the 5,7 position (Kong et al. 2004). In this paper, we report further details of the sex pheromone components of *D. houi*, and the results of laboratory and field tests of the pheromone.

Methods and Materials

Insects A laboratory colony of *D. houi* was started from cocoons collected in the field in Simao Prefecture, Yunnan Province. Pupae were sexed and allowed to emerge in a controlled environment room at 23±1°C, 60–80% hazard ratio (R.H.), and L16:D8 photoperiod. Female moths were used for pheromone extractions 1 or 2 d after emergence, and male moths were used for coupled gas chromatography–electroantennographic detection (GC–EAD) analyses.

Female Calling and Pheromone Collection The diel rhythm of calling behavior was observed for 1- to 4-d-old virgin females (N=126) in a controlled environment room. Observations were made under red light (3 l×) at 1-hr intervals during the scotophase.

Abdominal tips of virgin females including the pheromone gland were removed from 1- to 2-d-old calling females, 2–3 hr into the scotophase. The excised glands were extracted in 20 μl hexane for 20 min at room temperature, and then the extracts were transferred to a clean glass tube. Extracts from 6 or 15 abdominal tips were combined and concentrated to 2–3 μl by using a slow stream of N_2 before GC–EAD or GC–mass spectrometry (MS) analysis. If not used immediately, tubes were sealed and stored at −20°C.

Collections of airborne female sex pheromones were carried out by solid phase microextraction (SPME) with a polydimethylsiloxane fiber (100 μm coating; Supelco, Inc., Bellefonte, PA, USA). The fiber was conditioned in the GC injector (230°C) for 10 min before sampling. Single 1-d-old virgin females were transferred to a glass jar (250 ml) sealed with Parafilm, which was kept for 30 min in the controlled environment room mentioned above. Then the fiber was introduced into the headspace. After a 24-hr exposure, the fiber was retracted and the chemicals absorbed by the fiber were analyzed by GC as described below.

Chemicals Authentic standards were purchased from Chemtech B. V. (Amsterdam, the Netherlands) and purified in our laboratory by packed column GC (2 m×3.2 mm glass column, 8% PEG 20M on 80–100 mesh Chromosorb G-HP) described previously (Kong et al. 2001) to 99% purity for GC–EAD analysis, and > 95% for field tests. A standard solution containing the four stereoisomers of 5,7-dodecadien-1-ol (5,7-12:OH) and its corresponding acetates and aldehydes was prepared. A hydrocarbon standard solution (500 μg/ml in chloroform) was purchased from AccuStandard (New Haven, CT, USA).

Gas Chromatography–Electroantennographic Detection Gland extracts (15 female equivalents) were analyzed by GC–EAD with a Hewlett-Packard (HP) 5890 Series II GC equipped with a splitless injection system and a BP-20 column (25 m×0.25 mm i.d.× 0.25 μm film, SGE Pty. Ltd., Melbourne, Australia) programmed at 80°C for 1 min, then to 200°C at 10°C/min and held at 200°C for 30 min. The column effluent was split equally by an outlet splitter (SGE Co., part # OSS-2) between a flame ionization detector (FID) and an EAD male moth antennal preparation with 10 ml/min N_2 supplied as make-up gas. The EAD effluent was directed through a transfer line (250°C) to an L-shaped glass tube (7 mm i.d.), where it was mixed with purified humidified air (~ 500 ml/min), and then directed over the antennal preparation. The moth antenna was suspended between glass capillary electrodes filled with insect physiological saline (7.5 g NaCl, 0.21 g $CaCl_2$, 0.35 g KCl, 0.20 g $NaHCO_3$ per liter distilled water). The EAD responses were amplified tenfold with a probe amplifier, and data acquisition and analysis were performed with GC–EAD software (Syntech, Hilversum, the Netherlands).

Gas Chromatography and GC–Mass Spectrometry Analyses Pheromone gland extracts were analyzed by GC (Hewlett-Packard 6890 with flame ionization detector) and GC–MS (Finnigan Trace DSQ GC–MS, EI mode, 70 eV) with identification based on comparison of retention times and mass spectra with those of synthetic standards.

GC analyses were carried out on polar (DB-WAX, 30 m×0.25 mm i.d.×0.25 μm film; J&W Scientific, Folsom, CA, USA) and nonpolar (DB-5, 30 m×0.25 mm i.d.×0.25 μm film; J&W Scientific) columns with the injector at 200°C and oven temperature programmed from 80°C (1 min hold) to 220°C at 8°C/min, then held 10 min, using N_2 carrier gas. Introduction of chemicals from the SPME fiber onto the GC was by thermal desorption for 3 min in the injector at 200°C, then the analysis was performed under the same conditions as above. The DB-WAX column resolved the geometric isomers of

5,7-dodecadienal (5,7-12:Ald), 5,7-dodecadien-1-yl acetate (5,7-12:OAc), and 5,7-12:OH. The elution order of the isomers from the DB-WAX column was *ZE*, *EZ*, *ZZ*, and *EE*. For pheromone titer determination, individual pheromone gland extracts were prepared (N=21) and 4 ng (*Z*)-12-tetradecen-1-yl acetate (*Z*12-14:OAc) were added as internal standard (2 ng/μl). Absolute concentrations were calculated from the integrated peak area of each component relative to that of the internal standard.

GC–MS analyses were carried out on the DB-WAX column (as mentioned above), in which the full scan (41–460 amu) and selected ion monitoring (SIM) were recorded simultaneously. We scanned several intense and characteristic masses to ensure high sensitivity and selectivity. We selected *m/z* 224 (M^+), 164 (M^+-CH_3COOH), and 136 $\left(C_{10}H_{16}^+\right)$ to characterize the acetates of dodecadienols, *m/z* 182 (M^+) and 164 (M^+-H_2O) for dodecadienols, 180 (M^+) and 136 (M^+-CH_3CHO) for dodecadienals, 164 (M^+-H_2O) and 138 (M^+-C_2H_4O) for dodecenals, 166 (M^+-CH_3COOH) for dodecenyl acetates, 166 (M^+-H_2O) and 138 (M^+- C_2H_5OH) for dodecenols, and 238 (M^+) and 164 (M^+- CH_3CH_2COOH) for propionates of dodecadienols. The choice of these ions was based on mass spectra of the standards. The carrier gas was helium with a constant flow of 1.0 ml/min. Injector temperature was 220°C, and the split valve was off for 1 min. The temperature program was 60°C for 1 min, 6°C/min to 220°C, and hold for 10 min at 220°C. The GC-MS ion source and transfer line temperatures were 250°C and 220°C, respectively. After injection of six female-equivalent extracts, a reference sample was analyzed containing 5,7-diunsaturated C_{12} alcohols, aldehydes, acetates, (5*Z*,7*E*)-5,7-dodecadien-1-yl propionate (*Z*5,*E*7-12:OPr), and the monounsaturated compounds (*Z*)-5-dodecen-1-ol (*Z*5-12:OH), (*Z*)-5-dodecenal (*Z*5-12:Ald), and (*Z*)-5-dodecenyl acetate (*Z*5-12:OAc).

Field Tests Gray rubber septa (The West Company, Phoenixville, PA, USA), precleaned with ethanol, were treated with appropriate amounts of chemicals in hexane and then stabilized by adding butylated hydroxytoluene (BHT, 20 μg/μl) as antioxidant dissolved in dichloromethane (200 μg/septum). Controls were treated with 100 μl hexane and 200 μg BHT. Traps were placed in the field in three replications in 2004 (October 16 to November 17) and four replications each in 2005 (September 29 to November 8) and 2006 (June 19 to July 4) in the same pine forest near Simao city, Yunnan Province where cocoons had been obtained. Lures were left unchanged for the entire flight period. Sticky wing traps constructed from two pieces of cardboard (42×28 cm) were used in all field experiments. The septum was suspended on a wire at the center of the traps, 1–2 cm above the sticky trap bottoms. Virgin *D. houi* females in small mesh cages suspended in traps (N=4) were used as positive controls. Female moths were renewed every 4 d. Traps were deployed in a complete randomized block design. Traps were hung from branches of pine trees, about 1.5 m above the ground, at 15-m intervals. The numbers of moths in traps were checked and recorded at 1- to 2-d intervals, depending on weather conditions.

Data Analysis The data from field tests were analyzed by analysis of variance (ANOVA) after $\log(x+1)$ transformation. If significant effects were detected ($P<0.05$), LSD post hoc tests was used to separate differences between treatments. Kováts retention indices of each compound on each column were calculated from the formula for programmed temperature runs,

$$\mathrm{KI} = 100n + 100(N-n) \times \frac{\mathrm{Rt(unknown)} - \mathrm{Rt}(n)}{\mathrm{Rt}(N) - \mathrm{Rt}(n)},$$

where n and N are the carbon numbers of the *n*-alkane standards on either side of a given compound, Rt(unknown) is the retention time of the unknown, and Rt(n) and Rt(N) are the

retention times of the hydrocarbon standards (Marques et al. 2000). The KI data were calculated according to the retention times of GC–MS analyses, and the programmed temperature runs were performed under the same conditions as described above.

Results

GC–EAD and GC Analyses of the Gland Extracts of Females Three components in the crude gland extracts of *D. houi* elicited EAD responses, with component c (Rt: 14.08 min) giving the largest response (mean mV $\pm$ SD, $0.87 \pm 0.22, N = 6$) in all cases. Component b (Rt: 13.07 min) gave smaller but consistent responses (mean mV $\pm$ SD, 0.54 ± 0.12, $N = 6$), whereas component a (Rt: 11.15 min) gave small and less consistent responses (mean mV $\pm$ SD, $0.21 \pm 0.04, N = 4$) (Fig. 1). By comparing the GC–EAD retention times with those of synthetic standards, male moth antennae responded to *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald components, c, b, and a, respectively. The three components in the pheromone gland extracts that elicited EAD responses were further identified by comparison of retention times with those of reference compounds on both polar (DB-WAX) and nonpolar (DB-5) GC columns. On average, 14.7±12.9 ng (± SD) of *E*5,*Z*7-12:OH, 5.8±5.4 ng of *E*5,*Z*7-12:OAc, and 0.8±1.4 ng of *E*5,*Z*7-12:Ald were found in extracts, corresponding to a ratio of 100:39.7:5.6. The proportions of other isomers of the alcohol, acetate, and aldehyde in the sex pheromone gland extracts of *D. houi* were below the GC detection threshold, but were determined by SIM–GC–MS.

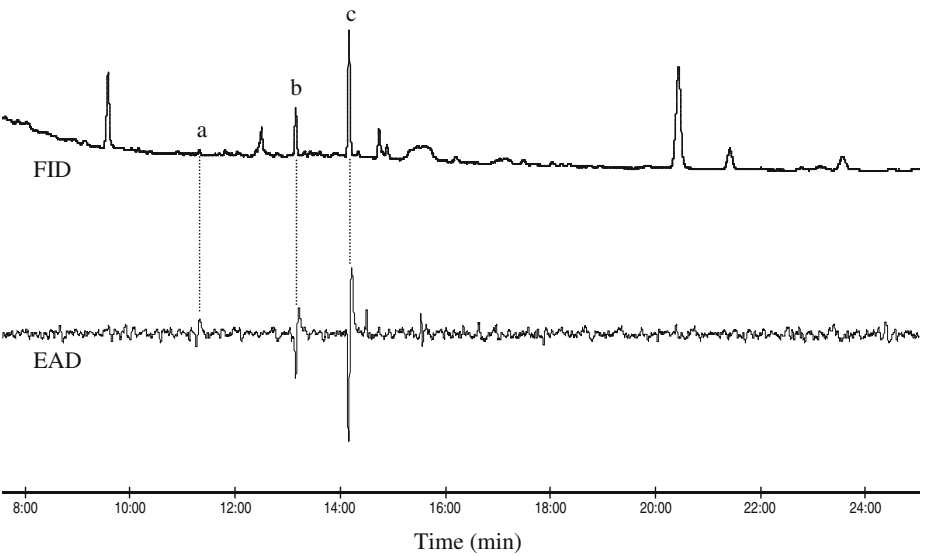


Fig. 1 Gas chromatography–electroantennographic detection (GC–EAD) traces from analysis of hexane extracts of the sex pheromone glands of *D. houi* virgin females (15 female equivalent; FID: flame ionization detector; EAD: male *D. houi* antenna). a, b, and c: FID peaks corresponding to electroantennography (EAG) responses. BP-20 column (see Methods and Materials)

Stereochemistry of the EAD-Active Components The retention indices of the three EAD-active components in solvent extracts (five female equivalents) from female pheromone glands on polar (DB-WAX) and nonpolar (DB-5) GC column were identical to those of synthetic *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald, and markedly different from the retention indices of the other isomers (Table 1). In SPME collections of pheromone components from live females, three major peaks were seen, two of which had retention times on DB-WAX coinciding with those of synthetic *E*5,*Z*7-12:OH (20.22 min) and *E*5, *Z*7-12:OAc (18.87 min). There was also a very small peak at the retention time of *E*5,*Z*7-12:Ald (16.51 min) (Fig. 2), and, the identity of this peak was verified from its mass spectrum. Complete resolution of the stereoisomers of all three EAD-active components substantiated the stereochemistry of the insect-produced compounds. The 5,7 position of the conjugated diene had been determined previously (Kong et al. 2004).

GC–MS Analyses of Gland Extracts of Females The presence of *E*5,*Z*7-12:Ald and its corresponding acetate and alcohol in extracts of female moths was confirmed with GC–MS analyses (Fig. 3). The identity of *E*5,*Z*7-12:OH as the major component of the pheromone of *D. houi* was indicated by the appearance of ions of *m*/*z* 182 (M^+, $C_{12}H_{22}O^+$, 7%) and 164 $\left(C_{12}H_{20}^+, 36\%\right)$ with a retention time of 22.87 min agreeing with that for the same ions and retention time of a synthetic sample of *E*5,*Z*7-12:OH. The spectra had other similarities including fragment ions at *m*/*z* 135 (M^+-C_2H_7O, 11%), 121 (M^+-C_3H_9O, 23%), and 79 $\left(C_6H_7^+, 81\%\right)$. The presence of *E*5,*Z*7-12:OAc in pheromone gland extracts was indicated by ions with *m*/*z* 224 (M^+, 18%), 164 (M^+-CH_3COOH, 49%), and 136 $\left(C_{10}H_{16}^+, 27\%\right)$ with a retention time coinciding with that for synthetic *E*5,*Z*7-12:OAc (21.39 min). Finally, the appearance of ions with *m*/*z* 180 (M^+, 13%) and 136 (M^+-CH_3CHO, 27%) at 18.47 min confirmed the presence of the *E*5,*Z*7-12:Ald. The retention behavior of authentic *E*5,*Z*7-12:Ald corresponded exactly with that of synthetic *E*5,*Z*7-12:Ald (18.47 min). In addition, other diagnostic fragment ions at *m*/*z* 162 (M^+-H_2O, 4%), 151 (M^+-CHO, 8%), and 79 $\left(C_6H_7^+, 100\%\right)$ were also observed within the mass spectra of the synthetic and insect-

Table 1 Kováts retention indices of four isomers of 5,7-dodecadien-1-ol and their corresponding acetates and aldehydes, and solvent extracts from female pheromone glands (5 fe) using db-wax and db-5 columns

Sample	Kováts Retention Index Values			
	DB-WAX Column		DB-5 Column	
	Standard	Solvent Extracts	Standard	Solvent Extracts
Z5,E7-12:OH	2,161		1,511	
E5,Z7-12:OH	2,168	2,167	1,514	1,514
Z5,Z7-12:OH	2,183		1,526	
E5,E7-12:OH	2,197		1,537	
Z5,E7-12:OAc	2,063		1,636	
E5,Z7-12:OAc	2,072	2,071	1,640	1,640
Z5,Z7-12:OAc	2,087		1,653	
E5,E7-12:OAc	2,108		1,666	
Z5,E7-12:Ald	1,884		1,436	
E5,Z7-12:Ald	1,896	1,895	1,442	1,441
Z5,Z7-12:Ald	1,909		1,452	
E5,E7-12:Ald	1,925		1,475	

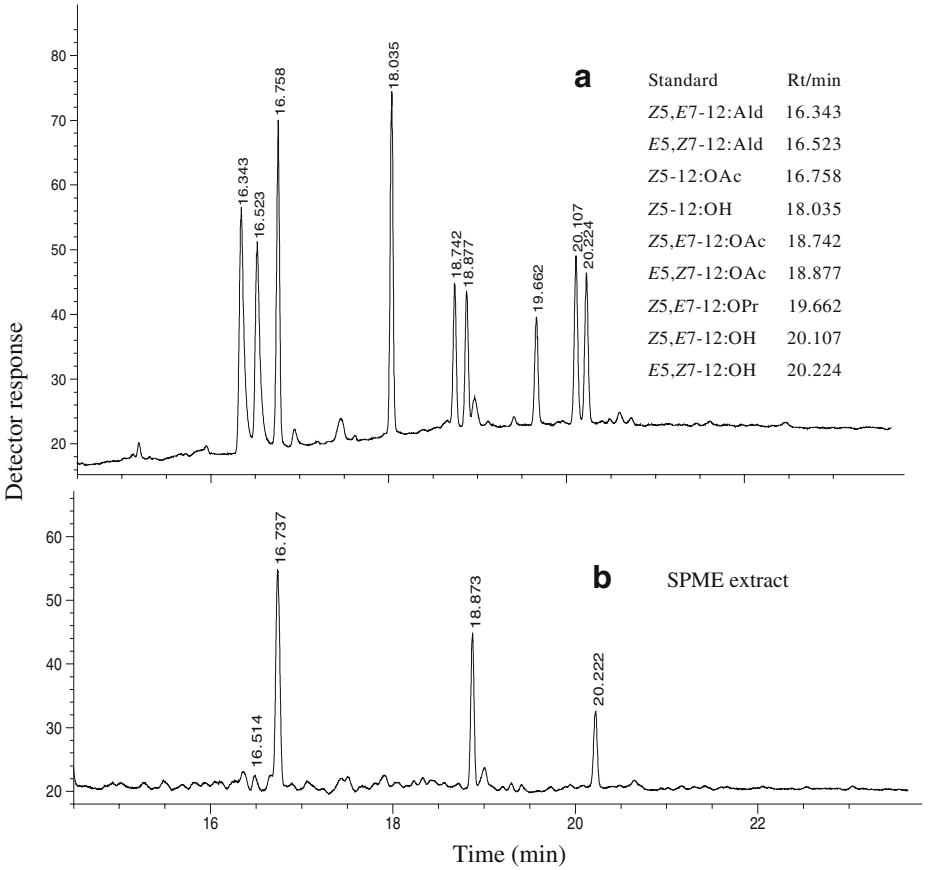


Fig. 2 Gas chromatograms of synthetic compounds (a) and a solid-phase microextraction (SPME) collection (b) of pheromone of *D. houi* using a DB-WAX column

produced (Fig. 3). Thus, the GC-MS information suggested that the pine moth sex pheromone gland extracts contained *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald.

To examine whether other pheromone-like compounds were present in the gland extracts, we monitored some characteristic ions, but did not detect *m*/*z* 164 (M^+-H_2O) and 138 (M^+-C_2H_4O) for dodecenals or 166 (M^+-CH_3COOH) for dodecenyl acetates at retention times corresponding to those of synthetic samples of *Z*5-12:Ald (15.73 min) and *Z*5-12:OAc (18.88 min). The same procedure excluded the presence of any dodecadienyl propionates as components of the gland extracts. However, the appearance in the mass spectrum of ions with *m*/*z* 166 (M^+-18, 6%) and 138 (M^+-46, 6%) (20.46 min) suggested the presence of a trace amount of *Z*5-12:OH (0.1% compared to that of *E*5,*Z*7-12:OH). In addition, according to diagnostic ions and retention time matches, we found possible trace amounts of (5*Z*,7*E*)-5, 7-dodecadien-1-ol (*Z*5,*E*7-12:OH, 0.7% compared to that of *E*5,*Z*7-12:OH) and (5*E*,7*E*)-5, 7-dodecadien-1-ol (*E*5,*E*7-12:OH, 0.7% to *E*5,*Z*7-isomer) by further examination of the reconstructed ion chromatogram of the female extracts. There were also possible trace amounts of *Z*5,*E*7-12:OAc (0.8% compared to *E*5,*Z*7-12:OAc), *Z*5,*Z*7-12:OAc (0.2% to *E*5,

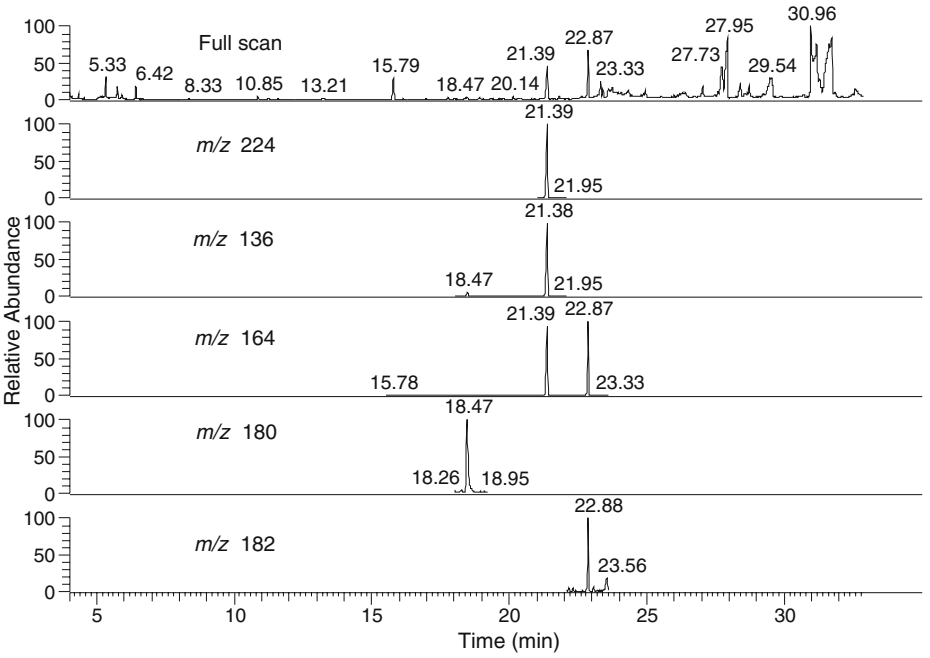


Fig. 3 Representative total ion and selected ion monitoring chromatograms of hexane extracts of calling female *D. houi* (6 FE). Analysis conditions are described in Methods and Materials

*Z*7-12:OAc), and *E*5,*E*7-12:OAc (0.8% to *E*5,*Z*7-12:OAc). No other isomers of *E*5,*Z*7-12:Ald were observed by GC–MS.

Field Tests In the first field test in 2004, the three components that elicited EAD response were not attractive to male *D. houi* moths as single components at doses of 1,000 μg, whereas lures with all binary combinations of *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald in the ratios found in analyses of pheromone gland extracts only resulted in a few captures over the second flight period. Traps baited with a 23:9.3:1 blend of alcohol/acetate/aldehyde were significantly more attractive than other treatments, suggesting that all three components were necessary for attraction (Table 2).

Optimization tests of the ratios of *E*5,*Z*7-alcohol, acetate, and aldehyde were conducted in 2005. In each of the three tests, the dose of *E*5,*Z*7-12:OH was held constant (5,000 μg/septum). In the first test (Table 3, experiment 1), lures loaded with a fixed ratio (5:1) of *E*5, *Z*7-12:OAc spiked with various amounts of *E*5,*Z*7-12:Ald all attracted similar numbers of moths. Increasing the amount of *E*5,*Z*7-12:Ald from 0.2% to 25% with respect to the major component, *E*5, *Z*7-12:OH, when *E*5, *Z*7-12:OAc was held constant at 2,000 μg/septum also had no effect on male moth catches (Table 3, experiment 2). However, increasing the amount of *E*5, *Z*7-12:OAc from 1.25% to 80% with respect to the major component, *E*5, *Z*7-12:OH, when *E*5, *Z*7-12:Ald was held constant at 250 μg/septum, showed a maximum catch at 5%, whereas other ratios were not different from each other (Table 3, experiment 3). These tests suggested that the ratio of *E*5, *Z*7-12:OAc to *E*5, *Z*7-12:Ald might be more important than the ratio of either component to *E*5, *Z*7-12:OH. Further field tests in 2006 were conducted to discriminate the effects of component ratios. In the first test, *E*5, *Z*7-12:OAc and *E*5, *Z*7-12:Ald were each held constant at 250 μg/septum, whereas the dose of *E*5,

Table 2 Catches of male *D. houi* in traps baited with potential pheromone components as single components, binary blends, and a ternary blend

Pheromone Component (μg)			Total Catch/Trap (Mean ± SE)[a]
*E*5,*Z*7-12:OH	*E*5,*Z*7-12:OAc	*E*5,*Z*7-12:Ald	
1,000	–	–	0
–	1,000	–	0.33±0.32 a
–	–	1,000	0
710	290	–	0.67±0.66 a
950	–	50	1.00±0.57 a
–	890	110	0.33±0.33 a
690	280	30	7.33±0.88 b
–	–	–	0

[a] Experiments were conducted in Simao City, Yunnan Province (three replicates, October 16–November 17, 2004). For ANOVA, data were transformed to log (x+1). Means followed by the same letter are not significantly different at the 5% confidence level by LSD test ($F_{(4,\ 10)}$=7.69, P<0.004).

*Z*7-12:OH was varied. All blends tested were equally attractive (Table 4, Experiment 1), suggesting that the ratio of *E*5,*Z*7-12:OH to the acetate and aldehyde was not as important as that of acetate to aldehyde. A second test was conducted in which *E*5,*Z*7-12:OH was held constant (5,000 μg/septum) and the amount of acetate and aldehyde (1:1) was varied. Increasing the amounts of the acetate and aldehyde relative to *E*5,*Z*7-12:OH had no effect on male moth captures (Table 4, Experiment 2). A final trial compared a synthetic pheromone blend (20:1:1) to virgin females (Table 4, Experiment 3). This three-component

Table 3 Catches of male *D. houi* in traps baited with *e*5,*z*7-12:OH and various amounts of *e*5,*z*7-12:oac and *e*5,*z*7-12:Ald

Pheromone Component (μg)			Total Catch/Trap (Mean ± SE)[a]
*E*5,*Z*7-12:OH	*E*5,*Z*7-12:OAc	*E*5,*Z*7-12:Ald	
Experiment 1			
5,000	62.5	12.5	5.25±2.06 a
5,000	250	50	8.00±1.73 a
5,000	1,000	200	6.00±0.74 a
5,000	4,000	800	6.50±1.85 a
Experiment 2			
5,000	2,000	10	8.50±2.47 a
5,000	2,000	50	5.50±1.32 a
5,000	2,000	250	6.25±2.25 a
5,000	2,000	1,250	4.25±1.81 a
Experiment 3			
5,000	62.5	250	8.00±1.41 a
5,000	250	250	15.50±2.02 b
5,000	1,000	250	7.75±1.93 a
5,000	4,000	250	5.75±1.25 a
–	–	–	1.25±0.60 c

[a] Experiments were conducted in Simao City, Yunnan Province (four replicates, September 29–November 8, 2005). For ANOVA, data were transformed to log(x+1). Means followed by the same letter are not significantly different at the 5% confidence level by LSD test (Experiment 1, $F_{(4,\ 15)}$=3.12, P<0.047; Experiment 2, $F_{(4,\ 15)}$=4.35, P<0.016; Experiment 3, $F_{(4,\ 15)}$=13.80, P<0.001).

Table 4 Catches of male *D. houi* in traps baited with three-component blends at various doses and ratios, and in comparison with virgin female moths

Pheromone Component (μg)			Total Catch/Trap (Mean ± SE)[a]
*E*5,*Z*7-12:OH	*E*5,*Z*7-12:OAc	*E*5,*Z*7-12:Ald	
Experiment 1			
5,000	250	250	26.50±6.22 a
1,000	250	250	27.75±2.78 a
200	250	250	26.25±5.85 a
40	250	250	30.25±5.53 a
Experiment 2			
5,000	10	10	33.25±2.48 a
5,000	50	50	17.75±4.42 a
5,000	250	250	28.50±5.83 a
5,000	1,250	1,250	34.00±4.24 a
–	–	–	2.75±2.13 b
Experiment 3			
5,000	250	250	31.25±3.75 a
Virgin female moth			22.75±2.28 a
–	–	–	1.50±0.28 b

[a] Experiments were conducted in Simao City, Yunnan Province (four replicates, June 19–July 4, 2006). For ANOVA, data were transformed to $\log(x+1)$. Means followed by the same letter are not significantly different at the 5% confidence level by LSD test (Experiment 1, $F_{(4,\ 15)}=13.09$, $P<0.001$; Experiment 2, $F_{(4,\ 15)}=15.36$, $P<0.001$; Experiment 3, $F_{(2,\ 9)}=160.71$, $P<0.001$).

blend was comparable to the attractiveness of virgin female *D. houi* moths (Table 4, Experiment 3).

Discussion

In view of the prevalence of *Z*5,*E*7-12:X as known sex pheromone components of the genus *Dendrolimus*, we tried the *Z*5,*E*7-aldehyde, alcohol, acetate, and propionate in preliminary field tests in 2001 and 2002, but none of these compounds in different combinations was attractive. In this paper, we presented evidence of the presence of three components, *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald in volatiles emitted by calling females and in hexane extracts of female sex pheromone glands of *D. houi*. When tested separately or in binary mixtures, *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald attracted a few *D. houi* males, but none of the binary blends was as attractive as the three component blend, indicating that all three components were necessary for optimal attraction. Further field tests confirmed the attractiveness of a blend similar to that found in pheromone gland extracts. However, further attempts to optimize the three-component blend were not straightforward. Further optimization may be warranted to find an effective blend while simultaneously minimizing the total dose of pheromone.

This is the first time that *E*5,*Z*7-isomers of alcohol, acetate, and aldehyde have been identified as sex pheromone components in *Dendrolimus* species. *ZE*- and *EZ*-isomers of 5,7-12:Ald, 5,7-12:OH, and 5,7-12:OAc may be involved as sex pheromone components in *Dendrolimus*, just as in *Malacosoma*, a related genus in which many species use *Z*5,*E*7- and *E*5,*Z*7-isomers in their sex pheromones. For example, *M. disstria* Hübner produced *Z*5,*E*7-12:Ald as a principal pheromone component and *Z*5,*E*7-12:OH as a minor component

(Chisholm et al. 1980). *E*5,*Z*7-12:Ald and *E*5,*Z*7-12:OH have been reported as sex pheromone components of *M. californicum* (Packard) (Underhill et al. 1980), *M. americanum* (F.) (Kochansky et al. 1996), and *M. neustrium* L. (Rotundo et al. 2004). However, *E*5,*Z*7-12:OH, *E*5,*Z*7-12:OAc, and *E*5,*Z*7-12:Ald have not been identified previously from any species in the genus *Dendrolimus*.

Dendrolimus houi and *D. kikuchii* are two closely related pine caterpillar species that cause major damage in pine forests in Yunnan province. Because *D. kikuchii* uses the *Z*5, *E*7-isomers of the acetate and alcohol components as its sex pheromone (Kong et al. 2001, 2005), differing pheromone chemistry provides a mechanism for reproductive isolation in these two sympatric *Dendrolimus* species. However, interspecific cross-attraction to synthetic pheromones of the two species was observed in field studies. Furthermore, the ratios and release rates of respective components also may be important for the two sympatric species to achieve separate communication channels. Other possible mechanisms may contribute to reproductive isolation between *D. houi* and *D. kikuchii*. For example, our laboratory investigations (unpublished) of the calling behaviors have shown that calling by *D. houi* peaks early into scotophase (~ 2 hr after dark) with a different periodicity than the other *Dendrolimus* spp. that we have investigated. In contrast, the peak of calling activity for *D. kikuchii* occurred 7–8 hr after the onset of scotophase. Thus, the different diurnal activity patterns suggest little overlap of female calling periods, which may assist in maintaining reproductive isolation of *D. houi* and *D. kikuchii*.

Structural similarities of pheromone components of *Dendrolimus* spp. may parallel phylogenetic relationships between species. The characteristics of the sex pheromones and sex attractants identified in *Dendrolimus*, together with genetic evidence, show that two groups of *Dendrolimus* species can be distinguished. The first group includes *D. pini* and *D. superans*, in which *Z*5,*E*7-12:Ald alone or in blends with the corresponding alcohol are attractive to males, whereas *Z*5,*E*7-12:OAc acts as an antagonist (Priesner et al. 1984; Kovalev et al. 1993; Kong et al. 2001). The second group includes *D. spectabilis*, *D. punctatus*, *D. tabulaeformis*, and *D. kikuchii*, in which the males are attracted by *Z*5,*E*7-12:OAc and its corresponding alcohol and/or propionate but inhibited by *Z*5,*E*7-12:Ald (Chen et al. 1980; Ando et al. 1982; Meng and Wang 1983; Kong et al. 2001, 2003). According to the sex pheromone blend identified in this paper, *D. houi* may represent the first of yet another group in *Dendrolimus*, which would provide an additional character for more precise grouping of the species within the genus. The differentiation of sex pheromones among closely related species may have been involved in the speciation process (Löfstedt 1993; Byers 2005, 2006). Further studies of the sex pheromones of other members of the genus and of pheromone-binding proteins in the antennae of male *D. houi* and *D. kikuchii* are in progress.

Acknowledgments The authors thank Lou Yong-gen for his critical review of the manuscript. We are also grateful to John A. Byers and Blanka Kalinová for their constructive comments and revision of the manuscript. Thanks are also due to Jocelyn G. Millar for technical assistance and advice. We are indebted to Liu Hong-ping, Tong Qing, and Mao Jia-ying and their staffs for assistance with field experiments. This work was supported by the National Natural Science Foundation of China (Grant No. 30300275).

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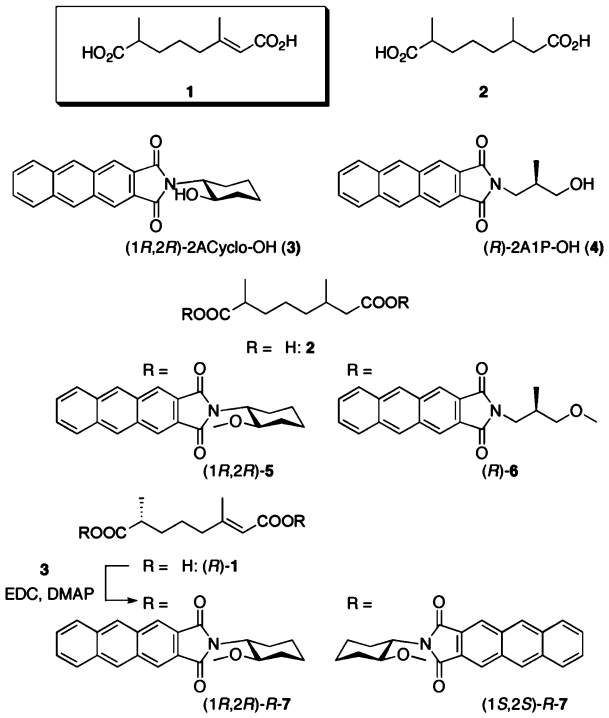


Fig. 1 Structures of the copulation release pheromone of *Callosobruchus chinensis*L. and *C. maculatus* and derivatization reaction

available. In an attempt to determine the absolute configuration of the natural product, both enantiomers of (**1**) were synthesized and assayed on male weevils (Mori et al. 1983). However, both enantiomers were active, and thus, the absolute configuration of the natural product was difficult to infer. Although several racemic (Tanaka et al. 1982; Kang and Lee 1987; Fontana et al. 1993; Wenkert and Khatuya 1999) and enantioselective (Gramatica et al. 1985; Giersch and Schulte-Elte 1990; Abo and Mori 1993; Yu et al. 2002; Nanda and Scott 2004) syntheses of (**1**) have been reported, the absolute configuration of the natural product has thus far remained obscure.

Recently, we reported the synthesis (Nakai et al. 2005) and determination of the stereoisomeric composition (Yajima et al. 2006) of the copulation release pheromone of the cowpea weevil, *C. maculatus*, [2,6-dimethyloctane-1,8-dioic acid (**2**)] (Nojima et al. 2007) using the Ohrui–Akasaka method (Akasaka et al. 1997, 1998; Akasaka and Ohrui 1999, 2004; Imaizumi et al. 2003). In this methodology, the crude natural product was derivatized directly to the corresponding Ohrui-Akasaka ester [(*bis*-2-(2,3-anthracenedicarboximido)-1-cyclohexyl (2ACyclo-O) ester (**5**) or *bis*-2-(2,3-anthracenedicarboximido)-1-propyl (2A1P-O) ester (**6**)] and analyzed by 2D-high-performance liquid chromatography (HPLC) without extensive purification. We found that the natural product is stereochemically impure, and the stereoisomeric composition of natural (**2**) was determined to be (2*R*,6*S*):(2*S*,6*R*):(2*S*,6*S*):(2*R*,6*R*)=43:38:18:trace (Yajima et al. 2006). This result indicated that it should be possible to establish the absolute configuration or stereoisomeric composition of natural (**1**) using the same method. As the Ohrui–Akasaka method employs a fluorescence detector instead of a UV detector for the HPLC analysis, femtomolar amounts of the material are sufficient for analysis. The absolute configurations of the biologically active natural products have been determined by using the Ohrui–Akasaka method (Akasaka et al. 2000, 2002; Morohashi

et al. 2000; Matsuhashi and Shimada 2002; Tashiro et al. 2002; Ohshiro et al. 2006). This technique has potential as a useful methodology for insect pheromone chemistry.

Methods and Materials

Azuki Bean Weevils, Callosobruchus chinensis L.

The weevils were maintained on azuki bean, *Vigna angularis* Dainagon, at 27°C and 60% relative humidity.

Collection and Fractionation of Copulation Release Pheromone, Callosobruchusic Acid

Collection was carried out according to Tanaka et al. (1981). A laboratory-maintained colony of *C. chinensis* L. was reared on azuki bean (*V. angularis*, Dainagon) at 27°C and 60% relative humidity in darkness. Newly emerged adults were sexed, and a group of several hundred virgin females was kept in a Petri dish (90 mm i.d.×60 mm height) with a pile of filter papers (40×20 cm) corrugated in 1-cm sections as shelters. After 20–27 d under these same conditions, the filter papers were Soxhlet-extracted with ether for 1 d. Extracts were combined, and the solvent was evaporated in vacuo. Extracts were dissolved in ether, and the solution was fractionated into neutral, acidic, and basic fractions with 5% NaOH aq and 5% HCl aq. The resulting acidic compounds were filtered through a short silica gel (1 g) column with hexane-ethyl acetate (30:1 to 0:100) as eluting solvent to remove highly polar compounds such as proteins or carbohydrates to give 7.0 mg of crude material.

Preparation of the Authentic Sample of (*R*)-(-)-Callosobruchusic Acid

Optically active (*R*)-(-)-callosobruchusic acid (**1**) was synthesized by using the method described by Mori et al. (1983). Based on ^{1}H NMR analysis of the synthetic intermediate, the enantiomeric purity of the synthetic **1** was estimated to be ca. 80% e.e. ^{1}H NMR spectra were recorded on a JNM-A400 spectrometer (Jeol Ltd., Tokyo, Japan). The spectral data of all the synthetic intermediates were in good accord with the reported data (Mori et al. 1983).

Preparation of Authentic *bis*-2-(2,3-Anthracenedicarboximido)-1-cyclohexyl (2ACyclo-O) Ester Derivative of the Synthetic **1** [(1*S*,2*S*)-R-**4** and (1*R*,2*R*)-R-**4**]

To a portion of synthetic (*R*)-**1** (ca. 1 mg, 5 mmol) in MeCN/toluene (1:1), (1*R*,2*R*)- or (1*S*,2*S*)- *bis*-2-(2,3-anthracenedicarboximido)-1-cyclohexanol [2ACyclo-OH (**3**), excess amount], 4-*N*,*N*-dimethylaminopyridine (DMAP, excess amount) and 2-(3-dimethylamino-propyl)-1-ethylcarbodiimide hydrochloride (EDC, excess amount) were successively added, and the mixture was stirred at 40°C for 24 hr. An aliquot was then loaded onto a silica gel TLC plate (10 cm length, Silica gel 60 F_{254}, Art-5744, Merck, Lindenplatz, Haar, Germany) and developed with hexane/EtOAc (4:1, *v*/*v*). The target spot, detected by fluorescence, was collected, packed in a Pasteur pipette and eluted with EtOAc/MeOH (5:1, *v*/*v*). After evaporation of the solvent with N_2 gas stream, the residue was purified by HPLC to give (1*R*,2*R*)-*R*-**4** or (1*S*,2*S*)-*R*-**4**. The HPLC conditions were as follows. Column: Develosil C30-UG-3 (4.6 mm i.d.×150 mm, Nomura Chemical Co., Aichi, Japan), mobile phase: MeCN/THF=9:1, temp: 20°C, flow rate: 1.0 ml/min. The structures of the synthetic samples were confirmed based on MS spectra recorded on a JMS-700 spectrometer (Jeol,

Ltd., Tokyo, Japan). The observed MS data were as follows: MS (FAB, positive): *m/z* 877 (8, M+Na), 832 (5), 803 (5), 441 (43), 413 (100), 391 (100), 328 (71), 279 (87), 225 (100), 223 (37); HRMS (FAB, positive): $[M+Na]^+$ 877.37 ($C_{54}H_{50}N_2O_8Na$; calc. 877.35).

Preparation of 2ACyclo-O Ester Derivative of the Natural Product

To a portion of the crude extract (ca. 1 mg, ca. 50 nmol) in MeCN/toluene (1:1), excess amounts of (1*R*,2*R*)- or (1*S*,2*S*)- 2ACyclo-OH (**3**), DMAP, and EDC were added successively, and the mixture was stirred at 40°C for 24 hr. An aliquot was then loaded onto a silica gel TLC plate (10 cm length, Silica gel 60 F_{254}, Art -5744, Merck) and developed with hexane/EtOAc (4:1, *v/v*). The target spot, detected by fluorescence, was collected, packed in a Pasteur pipette and eluted with EtOAc/MeOH (5:1, *v/v*). Partial evaporation of the solvent with N_2 gas stream gave (1*R*,2*R*)-*nat*-**4** or (1*S*,2*S*)-*nat*-**4**. The resulting sample was used directly for 2D-HPLC analysis.

2D-HPLC Analysis of 2ACyclo-O Ester Derivative [(1*S*,2*S*)-nat-**4** and (1*R*,2*R*)-nat-**4**]

To achieve complete separation of the target compounds from any impurities, we examined a two-dimensional HPLC (2D-HPLC) analysis method. 2D-HPLC in which two columns with different properties are used often results in improved separation (Erni and Frei 1978). The prepared (1*S*,2*S*)-*nat*-**4** or (1*R*,2*R*)-*nat*-**4** was injected using a Rheodyn 7125 loop injector (20 μl) and separated on two reverse-phase columns (first column: Develosil ODS-A-3, 4.6 mm i.d.×150 mm, second column: Develosil C30-UG-3, 4.6 mm i.d.×150 mm, Nomura Chemical Co., Aichi, Japan). Separation was performed by using two different solvent systems: first column, MeOH/H_2O=9:1 at a flow rate 0.6 ml/min (pump: CCPM, Tosoh Co., Tokyo, Japan) at 20°C (water bath); second column, MeCN/THF=76:24 (*v/v*) at a flow rate 0.6 ml/min (pump: PU-920, Jasco Co.) at 0°C (ice-water bath). The target fraction from the first column was injected into the second column using a six-way bulb system (PT-8000, Tosoh Co.). Detection was carried out by monitoring fluorescence intensity at 462 nm (excitation at 298 nm) using a FP-920 fluorescence detector (Jasco Co., Tokyo, Japan).

Results

Preparation of Authentic 2ACyclo-O Ester Derivatives [(1*S*,2*S*)-*R*-**4** and (1*R*,2*R*)-*R*-**4**]

As the (*S*)-isomer of synthetic **1** was not available, (1*S*,2*S*)- and (1*R*,2*R*)-2ACyclo-O derivatives of (*R*)-**1** [(1*S*,2*S*)-*R*-**4** and (1*R*,2*R*)-*R*-**4**] were prepared and subjected to analysis. In the preliminary experiment, both isomers were easily separated by using a single column (Fig. 2a) [Develosil ODS-A-3, MeCN/H_2O=9:1 (0.6 ml/min)] at 20°C (water bath). Retention times of the two isomers were 38.8 min [peak 1: (1*S*,2*S*)-*R*-**4**] and 52.8 min [peak 2: (1*R*,2*R*)-*R*-**4**], respectively. The separation factor α is estimated to be 1.39 (retention factors k': 13.9 and 19.3) for this separation of the enantiomers. As synthetic **1** was not enantiomerically pure, additional peaks were also observed on the chromatograms. Based on HPLC analysis, the enantiomeric excess of synthetic **1** was estimated to be 78.6–80.7% e.e. This result was in good accord with the ^1H NMR analysis of the synthetic intermediate. The major components were separated by HPLC to give pure samples of (1*S*,2*S*)-*R*-**4** and (1*R*,2*R*)-*R*-**4**.

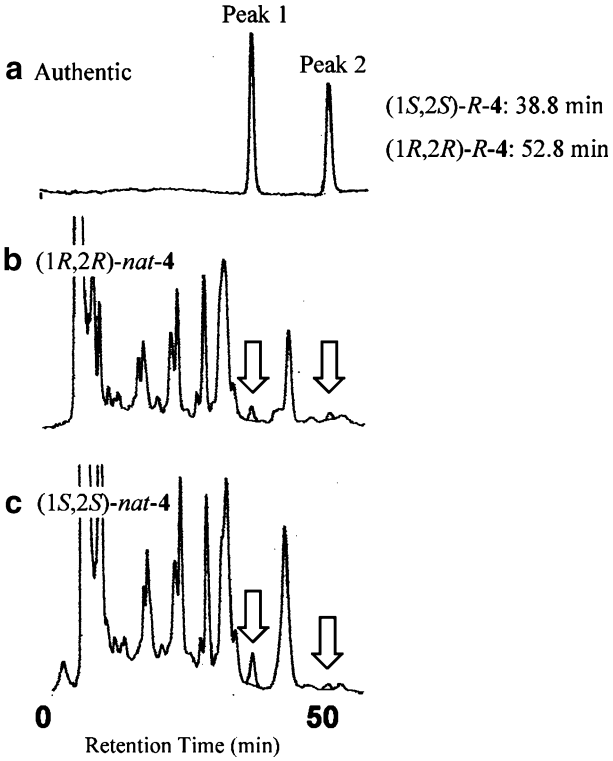


Fig. 2 HPLC chromatograms of 2ACyclo-O derivatives of synthetic callosobruchusic acid (a), (1*R*,2*R*)-*nat*-**4** (b), and (1*S*,2*S*)-*nat*-**4** (c). Peak 1: (1*S*,2*S*)-*R*-**4**; peak 2: (1*R*,2*R*)-*R*-**4**

Preliminary Analysis of 2ACyclo-O Ester Derivatives of the Natural Product [(1*S*,2*S*)-nat-**4** and (1*R*,2*R*)-nat-**4**]

In the same manner as for synthetic **1**, two portions (ca. 1 mg) of the crude extract were treated with (1*S*,2*S*)- and (1*R*,2*R*)-2ACyclo-OH to give the two isomers (1*S*,2*S*)-*nat*-**4** and (1*R*,2*R*)-*nat*-**4**, and these samples were subjected to HPLC analysis (Fig. 2b, c). The resulting chromatograms showed many peaks, but the target peaks 1 and 2, corresponding to those of (1*S*,2*S*)- and (1*R*,2*R*)-*R*-**4**, were observed. The peak area ratios of peaks 1 and 2 were 2.99:1 (panel B) and 6.31:1 (panel C). As the stereochemistry of the 2ACyclo-OH in the two samples were inverted with respect to each other, the peak area ratios should also have been inverted. This result indicated that the peaks did not consist of a single component, and therefore, some unidentified impurity was present.

2D-HPLC Analyses of 2ACyclo-O Derivatives

The Develosil C30-UG-3 column contains triacontyl alkyl chains and is doubly endcapped with trimethylsilyl groups, but the packing in the ODS-A-3 column does not share these properties. Because of their differing properties, the two columns were suitable for use in 2D-HPLC analysis. To avoid re-incorporation with impurities in the second column, only the target fraction, with a retention time of 36.6–39.9 or 50.7–54.6 min, was directly injected into the second column. To achieve this, a six-way bulb system (PT-8000, Tosoh Co.) was employed, and individual analysis of each target fraction was carried out. The chromatograms of the 2D-HPLC analyses of 2ACyclo-O derivatives are shown in Fig 3.

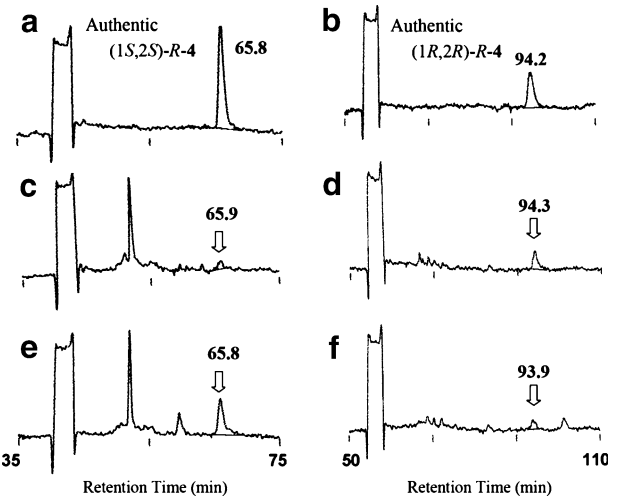


Fig. 3 Chromatograms of the 36.6- to 39.9-min fraction (a, c, and e) and the 50.7- to 54.6-min fraction (b, d, and f) obtained by 2D-HPLC analysis. (1*R*,2*R*)-*nat*-**4** (c and d); (1*S*,2*S*)-*nat*-**4** (e and f)

The overall retention times (over both columns) of the standard samples [(1*S*,2*S*)-*R*-**4** and (1*R*,2*R*)-*R*-**4**] were 65.8 min (peak 3, Fig. 3a) and 94.2 min (peak 4, Fig. 3b). Both isomers of **4** were observed in the chromatograms of the natural product derivatives (Fig. 3c, d, e, f). This result indicates that the natural callosobruchusic acid (**1**) is enantiomerically impure. The observed peak areas, which correspond to the stereoisomeric composition of the enantiomers of **1**, are listed in Table 1. Peak area ratios were in good accord with each other: peak 3/peak 4=1:3.38 (c and d) and 3.31:1 (e and f). As two enantiomers of 2ACyclo-OH were used for derivatization, the retention times of the two isomers of **4** were inverted with respect to each other. This accordance cannot be considered a coincidence. Based on the fact that the absolute configuration of the synthetic sample was *R*, the stereochemical composition of the natural callosobruchusic acid (**1**) was concluded to be *R*/*S*=3.3–3.4:1.

Discussion

As the identification of callosobruchusic acid (**1**) by Tanaka et al. (1981), several synthetic and biological studies of this pheromone component have been conducted. However, the absolute configuration of the natural product has heretofore remained obscure because of the scarcity and difficulty in purification of the natural product. The usual analytical method for chiral insect pheromones is GC analysis with a chiral stationary phase (Mori 1998). In the case of callosobruchusic acid (**1**), chiral GC analysis could not be carried out because

Table 1 Peak areas of 2ACyclo-O derivatives of the natural product in 2D-HPLC

Panel Fig. 3	2ACyclo-OH	Area		Absolute configuration (**1**)
		Peak 3	Peak 4	
B	1*R*,2*R*	1.39×10^5		*S*
C	1*R*,2*R*		4.70×10^5	*R*
D	1*S*,2*S*		7.67×10^5	*R*
E	1*S*,2*S*	2.32×10^5		*S*

the compound is not volatile enough to inject into a chiral column without decomposition. To overcome this problem, we used the Ohrui–Akasaka method. The natural pheromone was collected from 2,862 virgin female azuki bean weevils, and analysis was performed by using the unpurified crude sample. Although many peaks were observed on the resulting chromatograms, separation of the derivatives of the natural product was achieved using a 2D-HPLC technique. The natural pheromone was shown to be enantiomerically impure. As callosobruchusic acid (**1**) contains a carbonyl group next to a chiral center, accidental enolization of the carbonyl group can lead to racemization at C-2. However, by using a synthetic sample of **1**, it was shown that no racemization occurred during isolation, purification, or derivatization under the conditions used in this experiment. Thus, the ratio of enantiomers in the natural product was determined to be *R*/*S*=3.3–3.4:1. In studies of the relationship between stereochemistry and biological activity of **1**, Mori et al. (1983) found that (*R*)-**1** is just more than half as active as (*S*)-**1** [the dose necessary for 50% copulation release (ED_{50} value) for (*S*)-**1** was 6.5 ng, whereas that of (*R*)-**1** was 11.4 ng]. This means that female *C. chinensis* L. produce more of the less active enantiomer than the more active one. The reason for the lower level of production of the pheromone is unknown. The stereoisomeric composition of the copulation release pheromone of *C. maculatus* (**2**) was 2*R*,6*S*/2*S*,6*R*/2*S*,6*S*/2*R*,6*R*=43:38:18:trace, and there was a tendency for the more abundant isomer in the natural product to be more active (Yajima et al. 2006). We conclude that the biosynthesis and recognition of pheromones in weevils may not be strictly controlled.

Acknowledgments We wish to acknowledge the technical help provided by Professor K. Ohsawa, Professor S. Yajima, and Mr. K. Shimomura (Tokyo University of Agriculture) in the isolation of the pheromone.

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females, indicating that female moths continuously produce pheromone. A pheromone biosynthesis activating neuropeptide (PBAN)-like neuropeptide did not affect sex pheromone production as indicated by injection of synthetic PBAN and decapitation of *U. ornatrix* female adults. When the labeled precursor, D_4-9,12,15-18:acid, was injected into the early pupal stage, the most abundantly labeled hydrocarbons were 3,6,9-21:Hy and 1,3,6,9-21:Hy in the female adults. This result indicated that 3,6,9-21:Hy could be biosynthesized from linolenic acid through chain elongation and decarboxylation. To determine how 1,3,6,9-21:Hy is produced, D_4-3,6,9-21:Hy was injected into pupae and monitored for incorporation of label. No label was incorporated into 1,3,6,9-21:Hy, although a large amount of triene, 3,6,9-21:Hy, was recovered in the pheromone gland. This indicates that *U. ornatrix* females do not use 3,6,9-21:Hy to produce 1,3,6,9-21:Hy, and the terminal double bond is introduced earlier in the biosynthetic pathway.

Keywords Pheromone · Hydrocarbon · Biosynthesis · PBAN · Arctiidae · Lepidoptera

Introduction

One major group (type I) of moth sex pheromones is composed of linear fatty acid-derived compounds, 12–18 carbons in chain length, with an oxygenated functional group and one to three double bonds. A second major class (type 2) of sex pheromones is based on hydrocarbons with a polyene and/or epoxide functional group. This class of pheromones is typically found in four large moth families, the Geometridae, Noctuidae, Arctiidae, and Lymantridae (Byer 2006; Witzgall et al. 2004). The polyene type is biosynthesized from diet-derived linoleic or linolenic acids and characterized by 17–23 carbon chains with 1–4 double bonds, and 0–2 epoxides (Millar 2000).

Typically, type I sex pheromones are biosynthesized by specific enzymes in the pheromone gland and released by calling females (Jurenka 2003). In contrast, biosynthesis of type 2 occurs in specialized cells, oenocytes, that are associated with epidermal cells (Diehl 1975). The hydrocarbon pheromone of the arctiid moth, *Holomelina aurantiaca*, is biosynthesized in oenocytes and transported through the hemolymph to the pheromone gland for storage and release (Schal et al. 1998). In the lymantriid moth, *Lymantria dispar*, biosynthesis of the alkene precursor of the epoxide sex pheromone, occurs in oenocyte cells by chain elongation, desaturation, and decarboxylation. The alkene is transported to the pheromone gland and converted to (+)-disparlure by epoxidation (Jurenka et al. 2003). Polyene hydrocarbon precursors are present in the hemolymph of geometrid and arctiid moths, with the pheromone epoxide made in the pheromone gland (Wei et al. 2003, 2004).

Utetheisa ornatrix is broadly distributed over North America east of the Rockies and extends into South America, including Brazil, Argentina, and Chile (Pease 1968). Like many arctiid moths, *U. ornatrix* females produce a hydrocarbon pheromone and release it during the scotophase (Conner et al. 1980). The major sex pheromone component of *U. ornatrix* was identified as (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene (3,6,9-21:Hy). This component attracted male moths in the field (Conner et al. 1980). Subsequently, however, a different composition of sex attractant was found in this species, varying geographically within populations, and from year to year. This blend consisted of 3,6,9-21:Hy along with (*Z*,*Z*,*Z*)-1,3,6,9-heneicosatetraene (1,3,6,9-21:Hy) as a minor component (Huang et al. 1983; Jain et al. 1983). Recently, (*Z*,*Z*,*Z*)-3,6,9-eicosatriene 3,6,9-20:Hy has also been identified as a minor component produced by females of this species (Lim et al. 2007).

The male sex pheromone and related mating behavior of *U. ornatrix* has been studied comprehensively. After being attracted to the female, through the long distance sex

pheromone, the male courts the female by releasing hydroxydanaidal, derived from host plant pyrrolizidine alkaloids, obtained during larval feeding on seeds of *Crotalaria* spp. (Eisner and Meinwald 1995). The alkaloids are toxic, and female *U. ornatrix* may derive protection from predators by receiving these additional alkaloids during mating (Conner and Weller 2004). Because a female transfers some proportion of these alkaloids to her eggs, her offspring may also benefit (Iyengar and Eisner 1999; del Campo et al. 2005). Recent observations indicate that females appear to signal more vigorously in the presence of signaling neighbors, and they time their signaling in relation to their neighbors' signaling activity. This phenomenon has been described as "female pheromonal chorusing" (Lim and Greenfield 2007). This behavior indicates that females detect female-produced pheromone, as previously demonstrated by gas chromatography-electroantennographic detection (GC-EAD) studies (Grant and O'Connell 2000; Lim et al. 2007).

In the present study, by using gas chromatography-mass spectrometry (GC-MS) and GC-EAD analyses, we determined that 1,3,6,9-21:Hy is the major pheromone component in most females of a *U. ornatrix* colony originally collected from a population in Florida. However, about 10% of females in our colony produced large amounts of 3,6,9-21:Hy and no C-21 tetraene. By using deuterium-labeled fatty acid and hydrocarbon precursors, we determined that biosynthesis of 1,3,6,9-21:Hy occurred by chain elongation of linolenic acid. We further determined that a brain factor, such as the pheromone biosynthesis activating neuropeptide (PBAN), is not directly involved in regulating pheromone production.

Methods and Materials

Insects Colonies of *U. ornatrix* (Lepidoptera, Artiidae) were collected at the Archbold Biological Station (Highlands County, Florida, US). Larvae were reared on a pinto-bean diet mixed with seeds of the host plant, *Crotalaria mucronata* (Leguminoseae), as described (Dussourd et al. 1991) in an environmental chamber under a L:D14:10 at 26°C at the University of Kansas. Pupae were shipped to Iowa State University, where they were sexed and allowed to emerge separately. A sucrose solution (10%) was provided to adults. Virgin female adults or pupae were used throughout this study.

Chemicals (6,6,7,7-D_4)-9,12,15-octadecatrienoic (linolenic) acid (D_4-9,12,15-18:acid) was synthesized as described (Rakoff 1988). (12,12,13,13-D_4)-(*Z*,*Z*,*Z*)-3,6,9-heneicosatriene (D_4-3,6,9-21:Hy) was synthesized from D_4-9,12,15-18:acid, following procedures reported in Dogra et al. (1989). ^{1}H-NMR: δ 0.88 (3H, *t*, *J*=6.8 Hz, CH_3, C_{21}), 0.98 (3 H, *t*, *J*= 7.4 Hz, CH_3, C_1), 1.26–1.36 (18 H, *m*, CH_2, $C_{12–20}$), 2.04 (2 H, *d*, *J*=6.8 Hz, CH_2–C=, C_{11}), 2.08 (2 H, *m*, CH_2–C=, C_2), 2.80 (4 H, *t*, *J*=6.0 Hz, =C–CH_2–C=, $C_{5,8}$), 5.33–5.41 (6 H, *m*, CH=). ^{13}C-NMR: δ 14.11, 14.26, 20.54, 22.69, 25.52, 25.60, 27.00, 29.30, 29.35, 29.60, 29.64, 29.65, 29.66, 29.68, 31.93, 127.12, 127.56, 128.23, 128.30, 130.42, 131.94. EI-MS *m*/*z* (%): 294 $[M]^+$ (4), 265 (1), 238 (22), 184 (2), 170 (2), 149 (4), 135 (10), 121 (10), 108 (95), 95 (48), 93 (48), 79 (100), 67 (52), 55 (32). Synthetic *Helicoverpa zea* PBAN (Peninsula Laboratories, Belmont, CA, USA) was dissolved in an insect saline (21 mM KCl, 12 mM NaCl, 3 mM $CaCl_2$, 18 mM $MgCl_2$, 170 mM glucose, and 5 mM pipes buffer at pH 6.6 using KOH) before injection.

Incorporation and Hydrogenation Procedures The putative pheromone precursors, D_4-9,12,15-18:acid and D_4-3,6,9-21:Hy, were used to determine the biosynthetic pathway by injection directly into the hemocoel of pupae. Deuterium-labeled compounds were dissolved in vegetable oil and injected into 1- to 2-d-old female pupae. To investigate the

effect of PBAN, a saline solution with or without PBAN (10–50 pmol) was injected through abdominal intersegmental membranes into the hemocoel of 1- to 4-d-old decapitated females. Insects were incubated for 1 hr at room temperature, before the pheromone glands were removed, extracted, and analyzed for pheromones as described previously (Choi et al. 2002).

Hydrogenation of the unsaturated hydrocarbon pheromone, extracted from female pheromone glands, was conducted as follows. Hydrocarbon pheromone was purified by silica gel column chromatography. The purified hydrocarbon was dissolved in 100 μl of hexane in a glass bottle containing 10 mg palladium (5% wt on activated carbon; Sigma Chemical Co., St Louis, MO, USA). A hydrogen gas balloon covered the bottle for 2 hrs at room temperature before the hydrocarbon was extracted with hexane (100–200 μl), purified by silica gel column chromatography, and analyzed by gas chromatography (GC) and mass spectrometry (MS) as described below.

GC-EAD Analyses For GC-EAD analysis, a Hewlett-Packard 5890 Series II GC, equipped with a DB-5 column (30 m×0.25 mm ID, J & W Scientific, CA, USA) and a 50:50 effluent split, allowed simultaneous flame ionization detection (FID) and EAD of the gland extracts. The oven temperature program used was, 60°C for 1 min and increased to 250°C at 10°C min^{-1}. Helium was the carrier gas. Gland extracts were concentrated to 10–20 μl, under a gentle stream of nitrogen, and 1 μl was injected into the GC column in splitless mode. The outlet for the EAD was supplied with purified, humidified air flowing over the antennal preparation at 600 ml min^{-1}.

Whole-body preparations were used for EAD experiments. A virgin male or female moth was immobilized on a plasticine block with small U-shaped copper wires, and its antennae were restrained with thin copper wires. A glass microelectrode (tip diameter ≈ 1 mm, 0.86 mm I.D., A-M Systems Inc., WA, USA), filled with 0.1 M KCl, was introduced

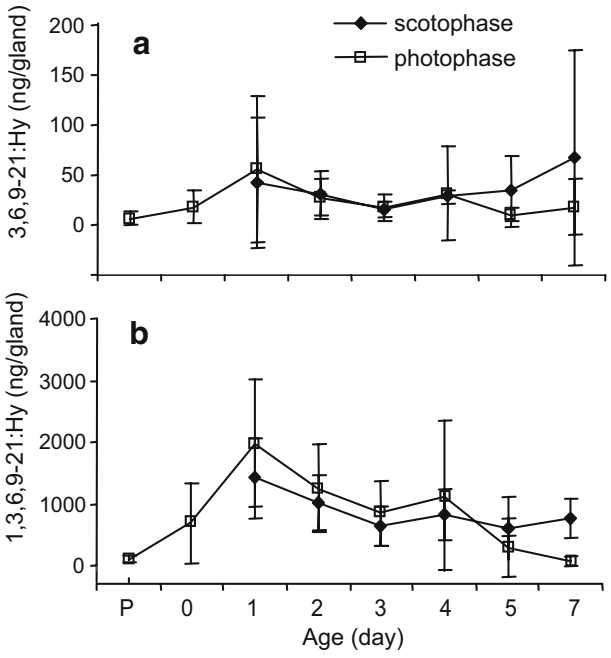


Fig. 1 Sex pheromone production by female *U. ornatrix* during the scotophase and photophase. **a** 3,6,9-21:Hy. **b** 1,3,6,9-21:Hy. *P* Pupae, *0* newly emerged female adult within 1 hr. *Values* represent the mean ± SD of at least ten replicates for each day

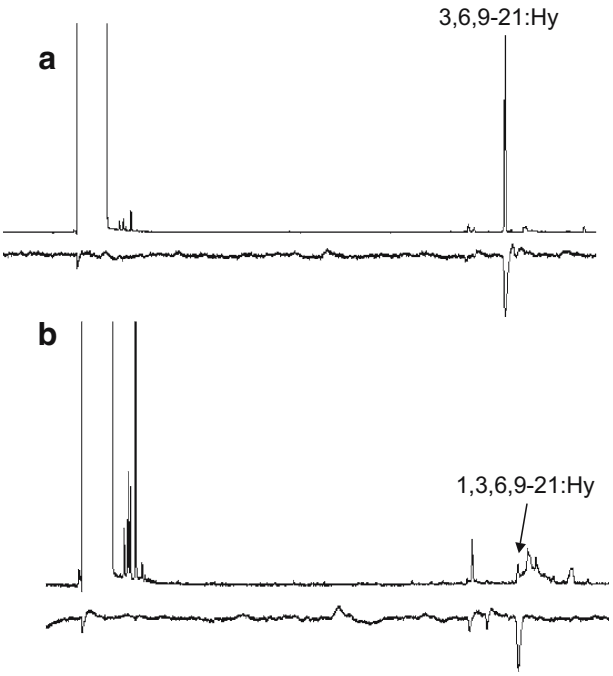


Fig. 2 GC-EAD responses of male *U. ornatrix* to female pheromone gland extracts. Extracts were obtained from a female producing 3,6,9-21:Hy (**a**) and from one producing 1,3,6,9-21:Hy (**b**). In both **a** and **b**, the *top traces* are GC-FID signals and *bottom traces* EAD responses. EAD-active compounds in the extracts were identified by GC and GC-MS

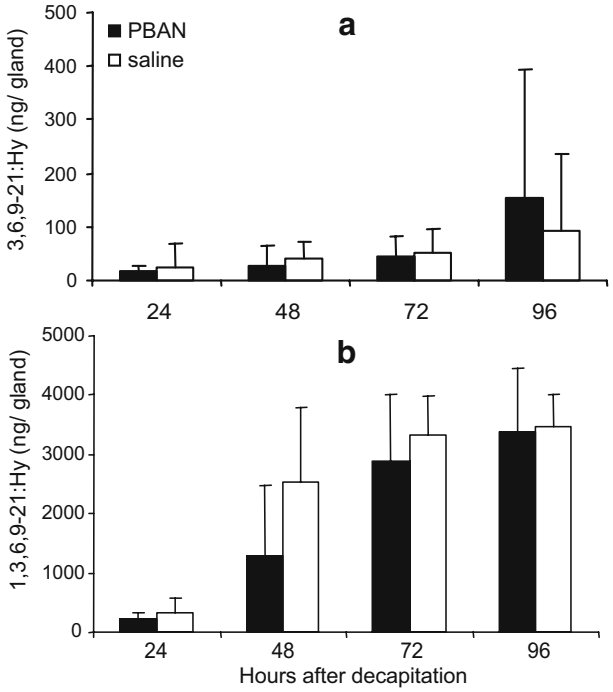


Fig. 3 Amount of pheromone components produced after injection of either saline or synthetic PBAN in decapitated females. **a** 3,6,9-21:Hy. **b** 1,3,6,9-21:Hy. One-day-old females were decapitated for 24–96 hr before injection. *Bars* represent the mean ± SD of at least ten replicates for each treatment

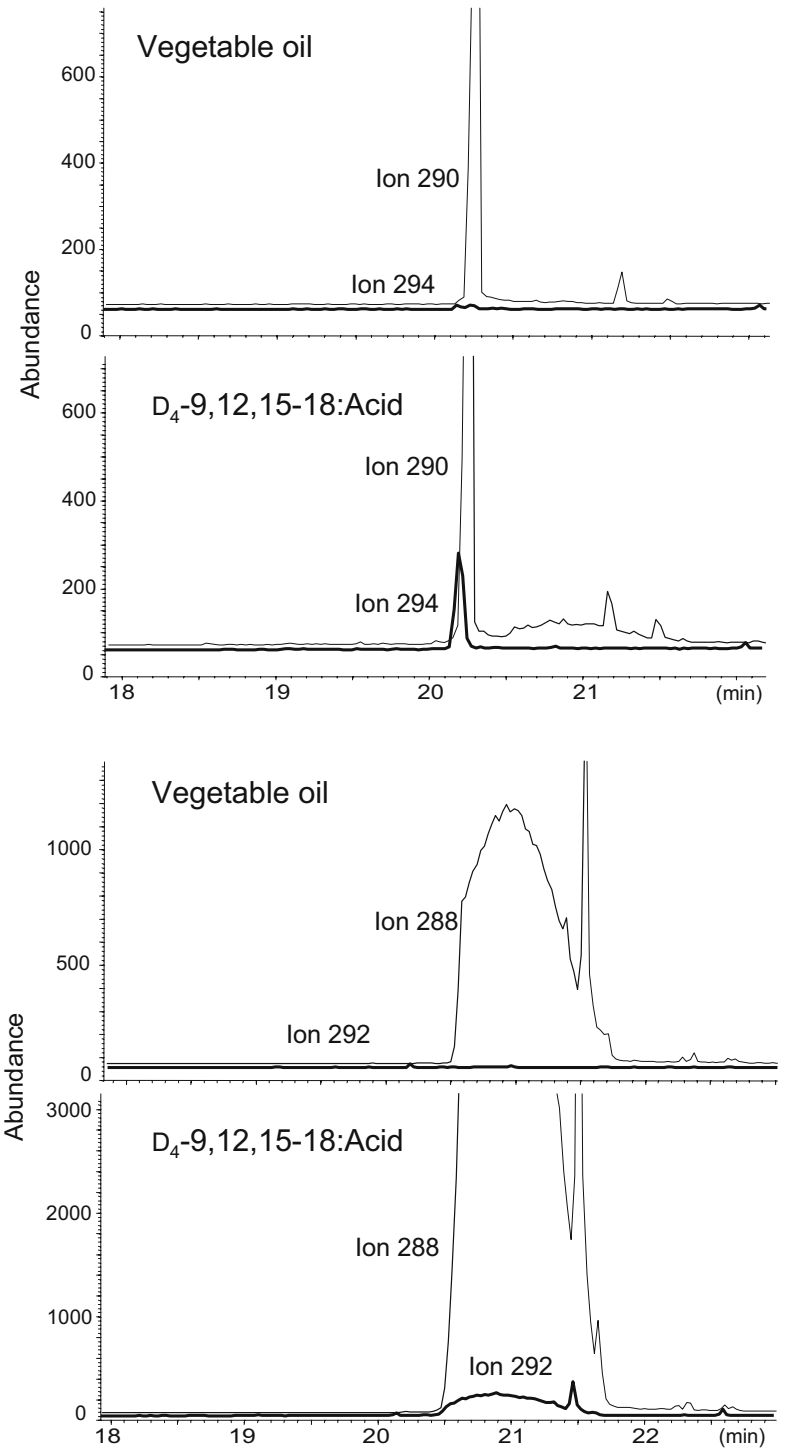

Vegetable oil
Ion 290
Ion 294
Abundance
D4-9,12,15-18:Acid
Ion 290
Ion 294
(min)
Vegetable oil
Ion 288
Ion 292
Abundance
D4-9,12,15-18:Acid
Ion 288
Ion 292
(min)

Fig. 4 Representative partial mass chromatograms obtained from GC-MS analysis of hexane extracts of pheromone glands of *U. ornatrix* after injection of 0.5 μl vegetable oil or 0.5 μl vegetable oil containing 50 μg D_4-9,12,15-18:acid into female pupae. The *top two chromatograms* were from females producing 3,6,9-21:Hy, and the *bottom two chromatograms* were from females producing predominantly 1,3,6,9-21:Hy. The GC-MS was set in the selective ion mode to detect *m/z* 288, 290, 292, and 294. Ions 292 and 294 are shown in *bold* and represent the label incorporation from the deuterium-labeled substrates

through the cuticle of the distal part of the antenna and served as a recording electrode; another glass microelectrode penetrated the moth's compound eye to serve as a reference electrode. Silver–silver chloride junctions were used to maintain electrical contact between the electrodes and amplifier. The electroantennogram signal was first amplified with a high-input impedance (>10 Ω) headstage preamplifier (Syntech®, Hilversum, The Netherlands), and further processed with a PC-based signal processing system (Syntech®).

Analyses by GC and GC-MS Glands were extracted with hexane (100 μl), containing 100 ng octadecane (18:Hy) as internal standard, for 10 min. Extracts were analyzed by GC or selected ion monitoring GC-MS. Incorporation of the labeled precursors into pheromones was determined by GC-MS with a Hewlett-Packard 5890 II mass selective detector coupled with a Hewlett-Packard 5890 GC equipped with a 30-m capillary column, DB-5 (30 m×0.25 mm; J&W Scientific, CA, USA). The oven was programmed from 50°C for 1 min, to 300°C at 10°C min^{-1}. The mass selective detector was used in the selected ion monitoring mode to detect the following characteristic *m/z*: 254 for octadecane (18:Hy), 276 for 3,6,9-20:Hy, 296 for heneicosane (21:Hy), 292 for 6,9-heneicoadiene, 290 for 3,6,9,-21:Hy, and 288 for 1,3,6,9-21:Hy. Labeled compounds were detected by using the characteristic ion +4. Adult females were extracted for pheromone and analyzed by GC-MS as described above.

Results and Discussion

Both 1,3,6,9-21:Hy and 3,6,9-21:Hy were detected in pheromone gland extracts of adults of all ages. The amounts of the two components showed a similar pattern with age between scotophase and photophase. Low amounts of both compounds were found in females during the late pupal stage (Fig. 1). The amount of 1,3,6,9-21:Hy varied greatly among individuals. The mean amount found in adult females was similar at all ages measured. However, the amount in 1-d-old females was greater than that in late pupae (Fig. 1B). The amount of 3,6,9-21:Hy (Fig. 1A) was similar for females of different age (including pupae), and roughly 1/30 that of 1,3,6,9-21:Hy (Fig. 1). These results indicate that sex pheromone biosynthesis starts in the late pupal stage, has increased by eclosion, and is maintained over at least 7 days. Thus, female adults are able to start calling and mate soon after emergence. This finding is consistent with previous work demonstrating that female adults are most active with respect to calling and mating at age 1- to 2-day-old (Conner et al. 1980; Itagaki and Conner 1987; Lim and Greenfield 2007).

GC analysis of pheromone gland extracts showed several peaks, including a very broad peak (Fig. 2B). Analysis indicated that all of these peaks, including the broad part of the chromatogram, had similar mass spectra, with the ion 288 prominent. The first peak was identified as 1,3,6,9-21:Hy based on comparison with published GC-MS data (Jain et al. 1983). This 21-carbon tetraene is unstable and the broad peak and peaks with longer retention times may result from thermolysis of this compound with rearrangement of double bonds into

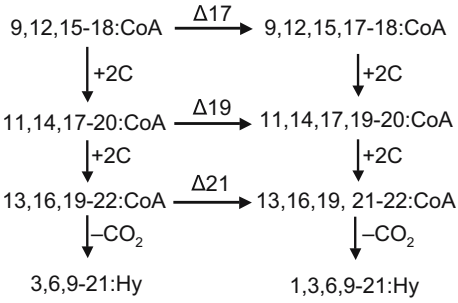


Fig. 5 Proposed biosynthetic pathways for production of the hydrocarbon sex pheromone components in *U. ornatrix*

conjugated configurations, such as 2,3,6,9-21:Hy. Hydrogenation of the extract resulted in the detection of only saturated 21:Hy. The areas of the peaks with longer retention times were also reduced if the GC temperatures for the injector and oven were decreased (unpublished data), supporting their formation from thermolysis of 1,3,6,9-21:Hy.

3,6,9-21:Hy, was initially identified as the major pheromone component of *U. ornatrix* females (Conner et al. 1980). In the present study, however, most females (~90%) produced about 1/30 the amount of this compound compared to 1,3,6,9-21:Hy. About 10% of female moths analyzed (several hundred) in our colony produced only a large amount (1,500–2,000 ng) of 3,6,9-21:Hy, without any tetraene (Fig. 2A). This result indicates that our colony may consist of two distinct pheromone types and that there may be two distinct populations present in the collection area in Florida. It has been noted previously that different geographical populations of *U. ornatrix* have different major and minor sex pheromone components (Conner et al. 1980; Huang et al. 1983; Jain et al. 1983). In their natural habitat, *U. ornatrix* host plants grow in highly populated patches. Presumably because of specificity to their hosts, *U. ornatrix* populations also show a clumped distribution. Such patchy distributions may allow for the formation of two pheromone types within a broader habitat. Further work is needed to determine the distributions of these two pheromonal types in populations in North and South America (Pease 1968).

Decapitated female moths appeared to accumulate 3,6,9-21:Hy and 1,3,6,9-21:Hy compared to same age normal females. Females, decapitated at 1-d-old, had roughly 3,300 ng of 1,3,6,9-21:Hy when 4-d old (Fig. 3), whereas 4-d-old intact females had roughly 1,000 ng of 1,3,6,9-21:Hy (Fig. 1). This result suggests that decapitated female moths continued to produce pheromone from oenocyte cells for transport to the pheromone gland, but probably did not release pheromone, as did intact females, due to the disconnect of the abdominal ganglia from the brain. The pheromone gland of female *U. ornatrix* consists of a long pair of slender tubes that extend forward to the sixth abdominal segment (Conner et al. 1980). Control of *U. ornatrix* rhythmic pheromone gland exposure by the terminal abdominal ganglion has previously been demonstrated (Itagaki and Conner 1987).

Although female *U. ornatrix* produce relatively large amounts of 1,3,6,9-21:Hy, only trace amounts were detected in the hemolymph during scotophase and photophase (unpublished data). This could be explained in several ways. First, transport of the hydrocarbon from the oenocytes to the pheromone gland may occur at a different time from when the hemolymph was collected. Second, the oenocyte cells are closely associated with the pheromone gland, and thus, transport through the hemolymph may not occur. Third, it is also possible that the pheromone gland directly produces the hydrocarbon pheromones. Further research will deduce which is the case.

A PBAN-like neuropeptide does not appear to influence hydrocarbon sex pheromone production in *U. ornatrix*, as indicated by similar amounts of pheromone being found in

decapitated females injected with either synthetic PBAN or saline (Fig. 3) and the increase in hydrocarbon quantities after decapitation discussed previously. PBAN is also not involved in hydrocarbon pheromone production in the noctuid moth, *Anticarsia gemmatalis*, (Jeong 2003). In other species of moths producing type 2 pheromones, PBAN regulates epoxidation after the uptake of hydrocarbons in the pheromone gland (Ando et al. 1997; Jurenka et al. 2003). Recently, it was shown that PBAN could regulate the uptake of hydrocarbon into pheromone glands of geometrid moths (Wei et al. 2004).

Fig. 4 shows results obtained from GC-MS analysis of pheromone gland extracts in which females were injected with labeled precursors. When D_4-9,12,15-18:acid was injected into the early pupal stage, the most abundantly labeled hydrocarbons in *U. ornatrix* adults were 3,6,9-21:Hy and 1,3,6,9-21:Hy. This indicates that 3,6,9-21:Hy could be biosynthesized by chain elongation and decarboxylation of linolenic acid. Typically, hydrocarbon pheromone biosynthesis occurs through modification of fatty acid metabolic pathways (Jurenka 2003). Labeling of the major pheromone component, 1,3,6,9-21:Hy, is of greater interest because it could be produced through two possible pathways based on when the terminal desaturation occurs (Fig. 5). To determine which pathway occurs, D_4-3,6,9-21:Hy was injected into pupae. No label was incorporated in 1,3,6,9-21:Hy, although a large amount of labeled triene, 3,6,9-21:Hy, was recovered. This suggests that *U. ornatrix* females do not use 3,6,9-21:Hy to produce 1,3,6,9-21:Hy. To determine when the terminal desaturation occurs in the biosynthetic pathway, additional studies will require the synthesis and injection of various chain-length precursors.

Acknowledgements This research was, in part, supported by the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, project 6698.

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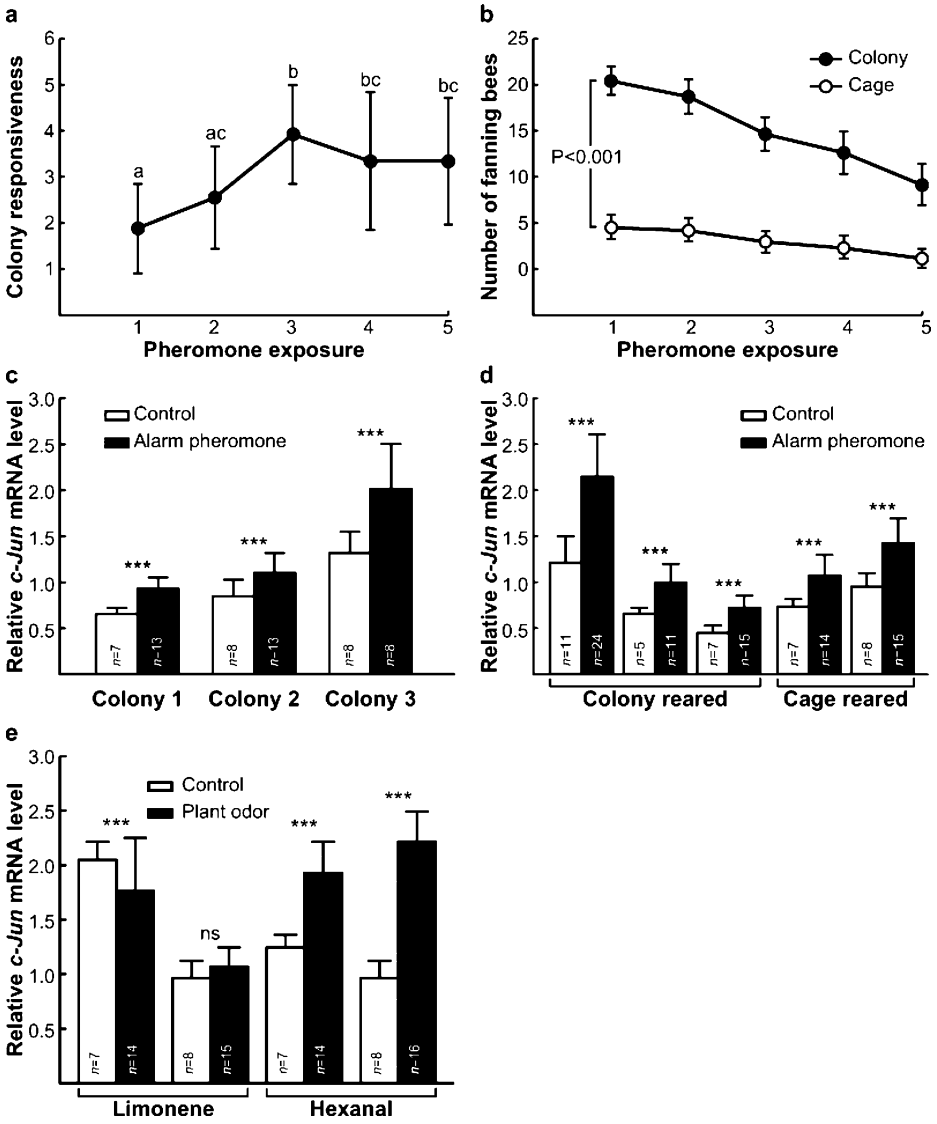
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We used isopentyl acetate (IPA), the principal component of the sting alarm pheromone in the honey bee, *Apis mellifera*, which elicits a response similar to that of the whole pheromone blend (Boch et al. 1962). Alarm pheromone provokes rapid defensive behavior, although longer-term effects are suggested by the observation that colonies that have been disturbed apparently remain aroused over a longer period (Winston 1987). Colony defense in honey bees typically begins with the release of alarm pheromone by "guard bees" at the hive entrance, which then provokes other bees to move to the entrance to face the intruder(s).

We first tested the effect of alarm pheromone exposure on longer latency behavior by repeatedly exposing bees to IPA in the field or laboratory. We then measured the effect of IPA exposure on the expression of the immediate early gene (IEG) and transcription factor, *c-Jun*, in the antennal lobes (ALs), the primary olfactory centers in the insect brain. Immediate early genes are involved in synaptic plasticity and are known to link experience

Fig. 1 Effect of exposure to alarm pheromone, or other odors, on honey bee behavior and *c-Jun* brain expression. **a** Repeated exposure to IPA caused a significant increase in colony arousal under natural conditions (two-way repeated measures ANOVA, colony: $F_{6,17}=81.92$, $P<0.001$, time exposure: $F_{4,68}=6.87$, $P<0.001$, interaction colony × time exposure: $F_{24,68}=0.88$, $P=0.621$). *Different letters* indicate significant differences in colony responsiveness over time (Tukey *post hoc* test, 1 vs. 3: $P<0.001$, 2 vs. 3: $P<0.01$, 1 vs. 4 and 5: $P<0.05$). Similar results were obtained for analyses based on number of bees responding relative to the first response (colony: $F_{6,17}=1.18$, $P=0.363$, time exposure: $F_{4,68}=3.09$, $P<0.001$, interaction colony × time exposure: $F_{24,68}=1.24$, $P=0.239$). The number of bees at the nest entrance ranged from 6 to 58 bees, and the mean lowest and highest responses were 30.7±13.5 and 65.4±23 bees, respectively. **b** Repeated exposure to IPA caused a significant decrease in responsiveness in the laboratory (rearing condition: $F_{1,18}=512.03$, $P<0.001$; time exposure: $F_{4,72}=94.31$, $P<0.001$; rearing condition × time exposure: $F_{4,72}=26.15$, $P<0.001$). Assays performed on caged bees reared either in typical colonies in the field or in laboratory cages (both $N=10$ cages). Isopentyl acetate-induced increase in *c-Jun* expression in the ALs of bees in typical colonies in the field (**c**) and laboratory cages (**d**). **e** Effect of exposure to known plant odors (limonene or hexanal) on *c-Jun* expression. Tests were performed as for IPA in the laboratory; colony-reared bees were exposed for 60 sec to 5 μl of neat odorant. We performed two trials, each with one control cage (filter paper alone) and two treatment cages, for each odor ($N=7$–8 individuals analyzed/cage). *Triple asterisks* represent $P<0.001$, Mann–Whitney rank sum test. Data show mean±SD. *Numbers in bars* show numbers of individual brains assayed

to changes in gene expression (Clayton 2000). The IEG *c-Jun* is expressed in neural circuits involved in olfaction in mammals (Baba et al. 1997).

Methods and Materials

Effect of Alarm Pheromone on Behavior Bees were exposed to a 60-sec presentation of 2.5 μl IPA, diluted in mineral oil (1/10) (Sigma-Aldrich, St Louis, MO, USA) (Collins and Rothenbuhler 1978), every 30 min ($N=5$ successive exposures). Mineral oil alone did not elicit a response (data not shown).

In the field assay, the pheromone solution was applied on a filter paper presented at the hive entrance ($N=7$ colonies, 2–4 replicates/colony, 24 replicates total). To measure colony arousal, the number of responding bees was determined from photographic snapshots of the hive entrance 0, 15, 30, 60, and 120 sec before and 15, 30, 60, and 120 sec after IPA exposure. Colony arousal was measured as the difference between the mean number of bees at the hive entrance before and after IPA exposure. The intensity of colony response at each exposure was ranked from lowest (rank 1) to highest (rank 5).

The laboratory assay was performed with cages of 25 8-d-old bees reared either in a typical colony in the field or in cages in the laboratory. Cage-reared bees were removed from their hive prior to eclosion by transferring honeycomb frames containing pupae to an incubator (33°C). They were then placed in cages upon eclosion and supplied with honey, pollen, and water. The cages were kept under a cardboard box (with holes on each side) at 32±2°C, 70–90% relative humidity (RH), and 12:12-hr light/dark photoperiod. Both colony- and cage-reared bees were transferred to test cages 4 hr prior to testing. This was done to minimize the effects of any exposure to alarm pheromone that might have occurred during transfer (IEG expression returns to basal levels 4 hr after stimulation; Clayton 2000). The IPA-treated filter paper was inserted under the wire floor of a Plexiglas cage (10×10×7 cm), and the wing fanning response was quantified. This assay has been shown to give responses that correlate with field behavior (Collins and Rothenbuhler 1978). Tests were conducted in a light room at 28±2°C and 30–40% RH.

Effect of Alarm Pheromone on IEG Expression *c-Jun* expression was analyzed 30 min after one exposure to IPA, a timescale appropriate for detecting an increase in IEG mRNA

(Clayton 2000). In the field experiment, bees standing at the hive entrance were randomly collected before IPA exposure (control); then, the colony was exposed to IPA for 60 sec, and the bees that rapidly approached the filter paper and behaved aggressively toward other bees were collected and placed in a Plexiglas cage for 30 min. In the laboratory experiment, bees exposed to either mineral oil (control) or IPA were frozen in liquid nitrogen 30 min after exposure. We performed three trials of the field assay, three trials of the laboratory assay with colony-reared bees (each one with one control cage and two treatment cages, N=5–12 individuals analyzed/cage), and two trials of the laboratory assay with cage-reared bees (each one with one control cage and two treatment cages, N=7–8 individuals analyzed/cage). Bees were held at −80°C until AL dissection and mRNA quantification by real-time quantitative reverse-transcriptase PCR (Grozinger et al. 2003). *c-Jun* mRNA levels were normalized to an mRNA loading control "housekeeping" gene (*rp49*). Primer sequences (5′ to 3′) were *c-Jun*, forward: CGTGGCGGCATCCAAA; *c-Jun*, reverse: CCCTTCAGCAATTTAACCTTATCTTC; (control gene) *rp49*, forward: GGAACT GGAAGTTTTAATGATGCA; and *rp49*, reverse: CAACAATGGATTTACGTTTT TTACTG. *c-Jun* expression was also determined following exposure to the known plant odorants, limonene and hexanal (Sigma-Aldrich).

Results and Discussion

Isopentyl acetate exposure affected subsequent behavioral responses of bees to alarm pheromone. There was a significant increase in the number of bees at the hive entrance over the first 1.5 hr before the response leveled off (Fig. 1a). Isopentyl acetate exposure also affected behavior in the laboratory but opposite to the field experiment; both colony- and cage-reared bees showed a significant decrease in responsiveness for at least 2.5 hr in response to IPA (Fig. 1b). The laboratory response of cage-reared bees was greatly attenuated relative to colony-reared bees (Fig. 1b). This is consistent with previous findings on the effects of queenlessness and social isolation on brain development (Gascuel and Masson 1987; Morgan et al. 1998).

The exposure of typical honey bee colonies in the field to IPA caused a significant increase in *c-Jun* expression (Fig. 1c). An equivalent *c-Jun* induction was also seen in response to IPA exposure in the laboratory, despite the opposite behavioral responses observed in the field and laboratory (Fig. 1a, b). The *c-Jun* response occurred equivalently in both colony- and cage-reared bees (Fig. 1d), although the behavioral response was stronger in colony-reared bees.

We tested the selectivity of this IEG response by exposing bees in cages to two other biologically relevant volatile odors: hexanal, a ubiquitous component of green leaf volatiles, and limonene, a floral odor. Hexanal induced *c-Jun*, but limonene did not (Fig. 1e). Speculation on the reasons for these contrasting results is beyond the scope of the present study; nevertheless, our results indicate that this particular IEG response in bees is not specific to alarm pheromone but is selective.

This is the first demonstration that a releaser pheromone can affect longer-latency behavioral response probabilities and cause changes in brain gene expression. These results demonstrate a genomic response to a releaser pheromone that is behaviorally related, although the genomic response appears to be less sensitive to behavioral context than does the behavior itself. *c-Jun* induction may initiate the response to alarm pheromone in ALs,

but integration with other mechanosensory and visual stimuli relevant to colony defense, present in the field but absent in the lab environment, apparently occurs at higher levels of the brain to shape the behavioral response (Li and Strausfeld 1999). Our findings blur the long-standing distinction between primer and releaser pheromones and highlight the pervasiveness of social regulation of brain gene expression (Robinson et al. 2005).

Acknowledgements We thank K. Pruiett for help with field work; T. Newman and M. Sen Sarma for primer design; T. Newman for assistance with molecular biology; and members of the Robinson lab and C.M. Grozinger, for comments on the manuscript. This work was funded by grants from the Fyssen Foundation to C.A. and the NIH and USDA National Research Initiative Program to G.E.R.

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thysanurans (Trembay and Gries 2003). Most of these insects inhabit enclosed microhabitats with little air movement, potentially rendering volatile pheromones less effective than contact pheromones. Moreover, many species that deploy contact pheromones for communication belong to primitive taxonomic insect orders (Grimaldi and Engel 2005). Identification of these chemicals will contribute to our understanding of pheromone evolution (Roelofs et al. 2002; Wertheim et al. 2005).

Here, we studied pheromonal communication in three primitive, synanthropic thysanurans, the common silverfish, *Lepisma saccharina* L., giant silverfish, *Ctenolepisma longicaudata* Escherich, and the firebrat, *Thermobia domestica* (Packard). Male, female, and nymph *T. domestica* produce, and respond to an aggregation pheromone (Trembay and Gries 2003) but require physical contact to perceive it. A similar communication system may exist in *L. saccharina* and *C. longicaudata*, as they also aggregate within human dwellings. Aggregating thysanurans maintain contact via antennae and caudal appendages (Tremblay 2002; NW, personal observation), suggesting that the pheromone may be associated with these or other body parts, although one cannot discount salivary secretions as yet another potential source. All three species can be found in the same shelter (NW, personal observation), and thus may be able to recognize con- and heterospecific pheromones.

Our objectives were to test the following hypotheses: 1) male, female, and nymph *L. saccharina* and *C. longicaudata* produce and respond to aggregation pheromone; 2) pheromone perception requires physical contact; 3) frass, scales, antennae, caudal filaments, or salivary secretions constitute the source of the *L. saccharina* pheromone; and 4) pheromones of *L. saccharina*, *C. longicaudata*, and *T. domestica* elicit a behavioral response by closely related heterospecifics.

Methods and Materials

Collection and Rearing of Experimental Insects

Thermobia domestica, *C. longicaudata*, and *L. saccharina* were collected by placing Petri dish (14×8 cm) traps baited with Quaker® oats on or around autoclaves and boilers at Simon Fraser University. Approximately 1,000 *L. saccharina* were also obtained from a laboratory colony at the University of Sussex. Insects were maintained in glass rearing jars (10×12 cm) provisioned with a moist cotton wick, a Petri dish (3×1 cm) containing Quaker® oats, and a corrugated cone of filter paper (CCOFP; Whatman® No. 1, 125-mm diam) as a shelter. Each jar housed either 100 males, females or juveniles, or a mixed group (100 insects total) of all stages. Jars were kept in a Plexiglas® container (30×22×22 cm) in contact with a heating rock (Zoo Med Labs, Inc., San Luis Obispo, CA, USA) so that the within-jar temperature ranged between 24° and 38°C depending on the proximity to the heat source. A within-jar relative humidity of 70–85% was maintained by leaving an open jar of water in contact with the heating rock. This range of humidity and temperature ensured optimal abiotic conditions for each of these species (Sweetman 1938, 1939; Spencer 1959). Insects were reared and tested under an 8L:16D photoperiod (Sweetman 1938, 1939).

General Bioassay Procedures

Lepisma saccharina, *T. domestica*, and *C. longicaudata* naturally reside within confined spaces with little or no air movement (Spencer 1930; Sweetman 1938). Thus, still-air "olfactometers" were used for all experiments. They consisted of a central Pyrex® glass

Petri dish connected to two lateral Petri dishes (each dish 9×3 cm) via tubing (2×2.5 cm) (Trembay and Gries 2003). Olfactometers were housed within opaque plastic bins (35×31×11 cm) (Columbia Plastics Ltd. ®, Vancouver, BC, CA), allowing diffuse but not directional light to enter. Each lateral chamber of an olfactometer received a filter paper (Whatman® No. 1, 125-mm diam) that was folded twice and formed into a cone, with the tip facing the central chamber. Cones served as shelters, resulting in few nonresponding individuals during bioassays (NW, personal observation). For each replicate, an insect (previously isolated for 16 hr) was released into the central chamber 6 hr after the onset of scotophase, and its position was recorded 16 hr later (2 hr before the start of scotophase). Each replicate was run at 24±2°C and 70–85% relative humidity (RH). Olfactometers were washed with hot water and Sparklene™ detergent, and were oven-dried at 125°C for 3 hr between each assay.

Specific Experiments

In experiments 1–9 and 10–18, we tested the hypothesis that male, female, and nymph *L. saccharina* and *C. longicaudata* produce and respond to species-specific pheromone. The CCOFP (see above) was removed from rearing jars after 3 d of exposure to 100 insects, brushed off to remove insect-derived frass and scales, and then cut into eight equal pieces. A single piece was then inserted into the paper cone located within the treatment chamber of each olfactometer. The paper cone in the corresponding control chamber received a piece of CCOFP that had been removed from rearing jars containing oats and a moist wick, but no insects.

In experiments 19–20 and 21–22, we tested the hypothesis that female *L. saccharina* (experiments 19–20) and *C. longicaudata* (experiments 21–22) require physical contact to perceive and respond to their aggregation pheromone. Considering that pheromone production and perception was neither gender- nor adult-specific in preceding experiments, this hypothesis was tested only with females. A CCOFP exposed for 3 d to a mixed group of male, female, and nymph conspecifics (100 insects total) was placed so as to be accessible (see above) or inaccessible in the treatment chamber of an olfactometer. Inaccessible stimuli were suspended above a nylon mesh, stretched over the rim of the chamber and thus out of reach for test insects. Control stimuli from rearing jars containing only oats and a moist wick were tested in a similar manner. One female *L. saccharina* or *C. longicaudata* was bioassayed in each replicate.

In experiments 23–26, we tested the hypothesis that *L. saccharina*-derived frass (experiment 23), body scales (experiment 24), antennae or caudal filaments (experiment 25), or salivary gland secretions (experiment 26) constitute the source of the aggregation pheromone. The test stimuli were prepared from: (1) dry fecal pellets (0.5 mg) recovered from female rearing jars after 3 d; (2) scales (0.5 mg) brushed off dorsal and ventral surfaces of 10 cold-anaesthetized females; (3) four antennae and six caudal filaments removed from four cold-anaesthetized females, or (4) macerated salivary glands excised from three cold-anaesthetized females. Each stimulus was spread within the paper cone shelter of a randomly assigned treatment chamber of an olfactometer. Paper cone shelters within the control chamber received no test stimulus. One female *L. saccharina* was bioassayed in each replicate.

In experiments 27–35, we tested the hypothesis that *L. saccharina*, *C. longicaudata*, and *T. domestica* respond to pheromones from con- and heterospecifics. Test stimuli consisted of a piece of CCOFP (see experiments 1–18) previously exposed to a mixed group of male, female, and juvenile insects. This was inserted into paper cones within the treatment

chamber of each olfactometer. Control chambers contained a piece of CCOFP from rearing jars containing only oats and a moist wick. One female *L. saccharina*, *C. longicaudata*, or *T. domestica* was bioassayed in each replicate.

Numbers of insects responding to test stimuli in each experiment were analyzed by χ^2 test, with JMP™ software (SAS®, Cary NC, USA). The significance level was set at $P \leq 0.05$. Insects found within the central chamber or connecting tunnels of olfactometers at termination of experiments were considered nonresponders and were not included in statistical analyses.

Results and Discussion

Male, female, and juvenile *L. saccharina* all exhibited significant arrestment responses to a piece of CCOFP previously exposed to either male, female, or juvenile conspecifics (Fig. 1). Similar results were obtained with *C. longicaudata* (Fig. 2). Female *L. saccharina* and *C. longicaudata* required physical contact with the pheromone to respond to it (Fig. 3). Neither dried insect-derived frass, body scales, antennae, caudal filaments, nor macerated salivary glands removed from females elicited significant arrestment responses by female *L. saccharina* (Fig. 4). Females of all species responded to conspecific pheromones, but varied in their response to those of heterospecifics. Female *C. longicaudata* were arrested by both heterospecific pheromones, female *L. saccharina* by neither, and female *T. domestica* responded only to that from *C. longicaudata* (Fig. 5).

The arrestant type pheromone of *T. domestica* (Trembay and Gries 2003), *L. saccharina* and *C. longicaudata* (this study) may be particularly suitable for marking potential shelters that provide appropriate microclimatic conditions (temperature, humidity), protection from predators, as well as access to food, water, and potential mates. We argue that increases in population density and consequently pheromone deposition may facilitate detection of suitable shelters, and may thus be correlated with the fitness of individual thysanurans. This correlation, an Allee effect (Stephens et al. 1999), is believed to be the driving force behind the evolution of aggregation pheromones (Wertheim et al. 2005) and may also help explain the increased survival of *T. domestica* nymphs when they were reared in groups rather than in isolation (Sweetman 1938).

Nonvolatile insect pheromones identified thus far are discrete or complexed fatty acids (Naumann et al. 1991; Finidori-Logli et al. 1996; Kugimiya et al. 2002), saccharides

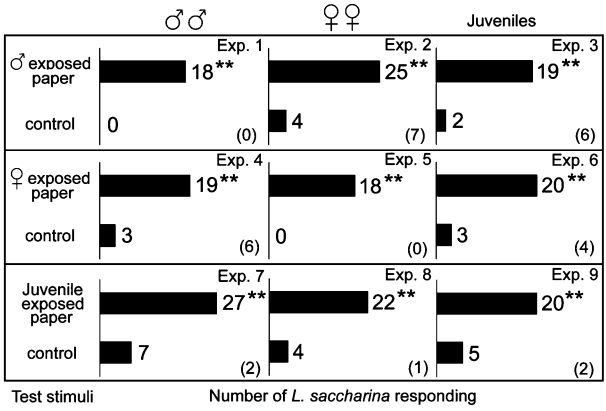


Fig. 1 Number of male, female, or juvenile *L. saccharina* responding to a piece of filter paper previously exposed to male, female, or juvenile *L. saccharina*. Numbers near bars indicate the number of insects responding to the test stimulus. An asterisk (*) indicates a significant preference for a particular test stimulus (χ^2 test; $^*P<0.05$, $^{**}P<0.01$). Numbers in brackets indicate numbers of nonresponding insects

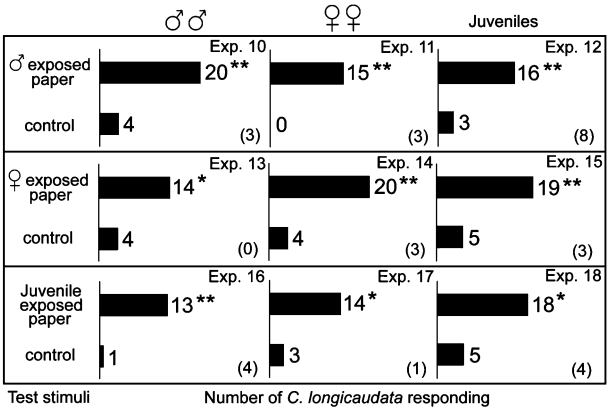


Fig. 2 Number of male, female, or juvenile *C. longicaudata* responding to a piece of filter paper previously exposed to male, female, or juvenile *C. longicaudata*. Numbers near bars indicate the number of insects responding to the test stimulus. An asterisk (*) indicates a significant preference for a particular test stimulus (χ^2 test; $^*P<0.05$, $^{**}P<0.01$). Numbers in brackets indicate numbers of nonresponding insects

(Nojima et al. 1999, 2002), or steroids (Sakuma and Fukami 1993; Kugimiya et al. 2003). All of these pheromones are easily extracted with solvents of polarity similar to that of the pheromones themselves. Our preliminary attempts to extract thysanuran pheromones with diverse polar and nonpolar solvents failed, suggesting that thysanuran pheromones may possess both polar and nonpolar segments. Such amphipathicity would allow micellar formation or polymerization, and adherence of the pheromone to solid substrates. Two amphipathic steroidal glucoside pheromones (Blattellastanoside-A and Blattellastanoside-B) have been isolated from the German cockroach, *Blattella germanica* (Sakuma and Fukami 1993). Similar to thysanuran pheromones, they are produced by males, females, and nymphs, are perceived upon contact, and elicit arrestment (Ishii and Kuwahara 1967, 1968).

The nonvolatile and nonlabile nature of thysanuran pheromones make them suitable as aggregation and shelter markers. Such pheromones resist evaporation even at temperatures of 27–38°C, the range required by *C. longicaudata* and *T. domestica* (Adams 1959; Spencer 1959). Thus, the pheromone would not saturate the confined space typically inhabited by thysanurans, nor cause adaptation and habituation of their olfactory system. Moreover, a nonpolar or amphipathic pheromone does not readily dissolve in the humid microclimate (55–97% RH) that thysanurans require to prevent desiccation (Sweetman 1938, 1939; Adams 1959).

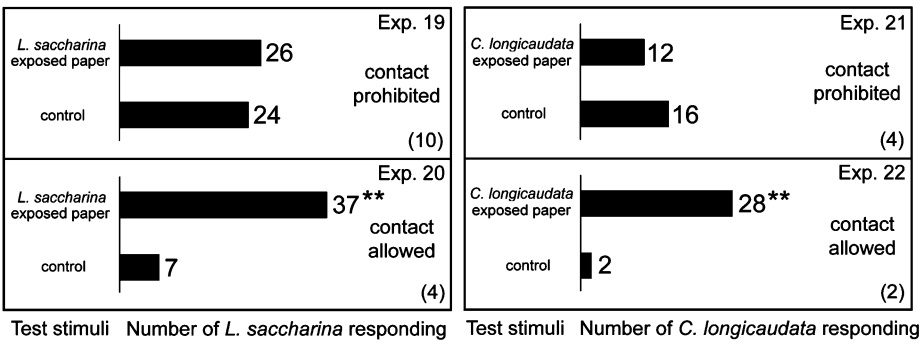


Fig. 3 Number of female *L. saccharina* (left) or *C. longicaudata* (right) responding to a piece of filter paper previously exposed to male, female, or juvenile conspecifics when stimulus contact was prohibited or allowed. Numbers near bars indicate the number of insects responding to the test stimulus. An asterisk (*) indicates a significant preference for a particular test stimulus (χ^2 test; $^*P<0.05$, $^{**}P<0.01$). Numbers in brackets indicate numbers of nonresponding insects

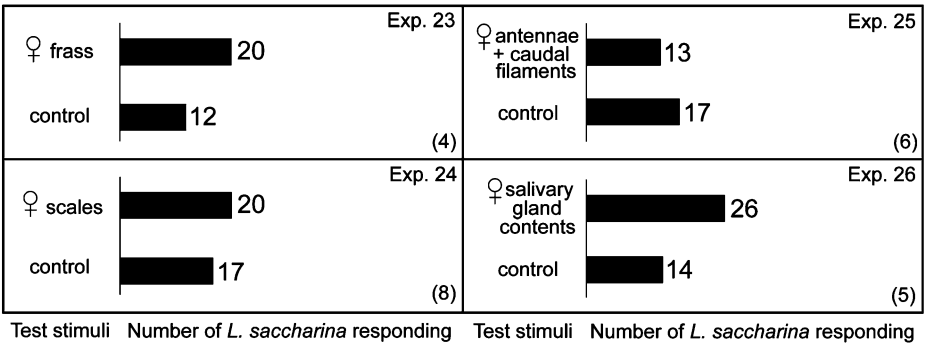


Fig. 4 Number of female *L. saccharina* responding to conspecific frass, scales, antennae and caudal filaments, or salivary gland contents. Numbers near bars indicate the number of insects responding to the test stimulus. In each experiment, there was no significant preference for a test stimulus (χ^2 test; $P<0.05$, $^{**}P<0.01$). Numbers in brackets indicate numbers of nonresponding insects

The aggregation pheromones of *L. saccharina*, *C. longicaudata* (Figs. 1 and 2), and *T. domestica* (Trembay and Gries 2003) are produced by females, males, and nymphs, suggesting they are derived from a source common to nymphs and adults. However, none of the potential pheromone sources tested previously (Trembay and Gries 2003) or in this study (Fig. 4) elicited significant arrestment in any of these species. Frass and macerated salivary glands appear to have caused some arrestment responses (Fig. 5) and ought to be investigated further, particularly in light of reports that the aggregation pheromone of European earwigs, *Forficula auricularia*, is present in frass and shed cuticle (Walker et al. 1993) or tibial glands (Sauphanor 1992). We also investigated the possibility that the arrestment behavior was caused merely by insect-induced physical and chemical changes of the shelter, or food sources therein. However, we dismissed this possibility, as glass surfaces exposed to insects for several days in the absence of food elicited strong arrestment by conspecifics (data not shown), clearly indicating that the insects deposit signals.

Relatedness between *C. longicaudata*, *T. domestica*, and *L. saccharina* can be investigated based on diverse criteria. Morphologically, *C. longicaudata* and *T. domestica* are more similar to each other than they are to *L. saccharina*. Arrestment response by *C. longicaudata*, but not *L. saccharina*, to the *T. domestica* pheromone (Trembay and Gries 2003), and lack of arrestment response to heterospecific pheromone between *T. domestica*

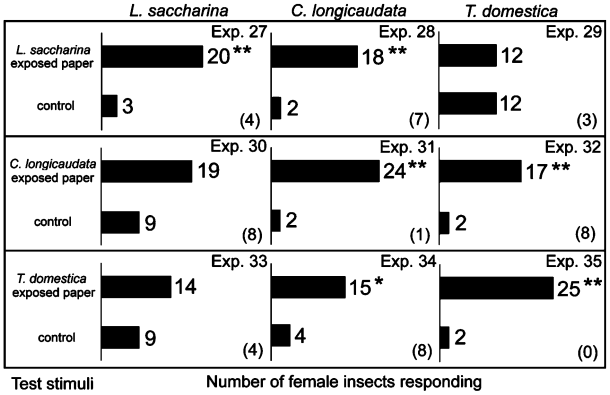


Fig. 5 Number of female *L. saccharina*, *C. longicaudata*, or *T. domestica* responding to a piece of filter paper previously exposed to conspecific or heterospecific male, female, and juveniles. Numbers near bars indicate the number of insects responding to the test stimulus. An asterisk (*) indicates a significant preference for a particular test stimulus (χ^2 test; $^{*}P<0.05$, $^{**}P<0.01$). Numbers in brackets indicate numbers of nonresponding insects

and *L. saccharina* (Fig. 5), all support the idea that *C. longicaudata* and *T. domestica* are more closely related to each other than they are to *L. saccharina* (Mendes 1991). A definitive conclusion, however, must await analyses of all three species by molecular genetics. The fact that *C. longicaudata* females respond to both *L. saccharina* and *T. domestica* pheromone (Fig. 5) is somewhat surprising, but may be explained by the probability of co-inhabiting a shelter with one of these heterospecifics. *Lepisma saccharina* and *T. domestica* prefer shelter temperatures between 21–27°C and 27–43°C, respectively (Spencer 1930, 1959; Sweetman 1938, 1939), and by selecting such discrete temperature regimes they are effectively isolated. In contrast, *C. longicaudata* is readily found in shelters at temperatures between 17°C and 30°C (Lindsay 1940), overlapping those of both *L. saccharina* and *T. domestica*. Thus, *C. longicaudata* may frequently encounter suitable shelters of either *L. saccharina* or *T. domestica* and may have evolved the ability to recognize their respective pheromones.

Acknowledgments We thank Dave Booth for supplying *Lepisma saccharina*, Michelle Tremblay for advice on the capture and rearing of all thysanuran species, Robert Britton, Andrew Lewis, Allen Haddrell, Regine Gries, Grigori Khaskin, and Aleksander Miroshnychenko for help and advice regarding preliminary chemical analyses, Kate Mclellan, and all laboratory colleagues for their advice throughout this project, as well as Jeremy McNeil and two anonymous reviewers for constructive comments. The research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC)-Industrial Research Chair to G.G. with SC Johnson Canada, Pherotech International Inc. and Global Forest Science (GF-18-2007-226 and GF-18-2007-227) as the industrial sponsors.

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Table 1 Oligonucleotide primers used for isolation of *Scleroderma guani* odorant-binding and chemosensory proteins (OBPs and CSPs)

Purpose/Primer Name	Sequence (5′–3′)
cDNA Isolation (RT-PCR)	
Oligo dT-3 site Adaptor Primer	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTVN
3′ cDNA End Isolation (RACE)	
SguaOBP1-S1 (sense)[a]	CTNTCNGARGCNGAYGT
SguaOBP1-S2 (sense)[a]	CTNAGYGARGCNGAYGT
SguaOBP1-S3 (sense)[a]	TTRTCNGARGCNGAYGT
SguaOBP1-S4 (sense)[a]	TTRAGYGARGCNGAYGT
SguaOBP2-S1 (sense)[b]	GCNGAYAAYGAYGAYCC
SguaCSP1-S1 (sense)[c]	ACNCCNGARTAYTAYAC
Adapter Primer (antisense)	CTGATCTAGAGGTACCGGATCC
5′ cDNA End Isolation (RACE)	
SguaOBP1-AS1 (antisense)[d]	GACACCTTGGTCGTTCATCATAC
SguaOBP2-AS1 (antisense)[d]	TGGCACGGCTCACTGACTGTTA
SguaCSP1-AS1 (antisense)[d]	GAACACTTGGAACATACCGTCTCT
Outer Primer (sense)	GCTGATGGCGATGAATGAACACTG

[a] Corresponds to N-terminal region amino acid sequence: LSEADV.

[b] Corresponds to N-terminal region amino acid sequence: ADNDDP.

[c] Corresponds to N-terminal region amino acid sequence: TPEYYT.

[d] Gene-specific primer

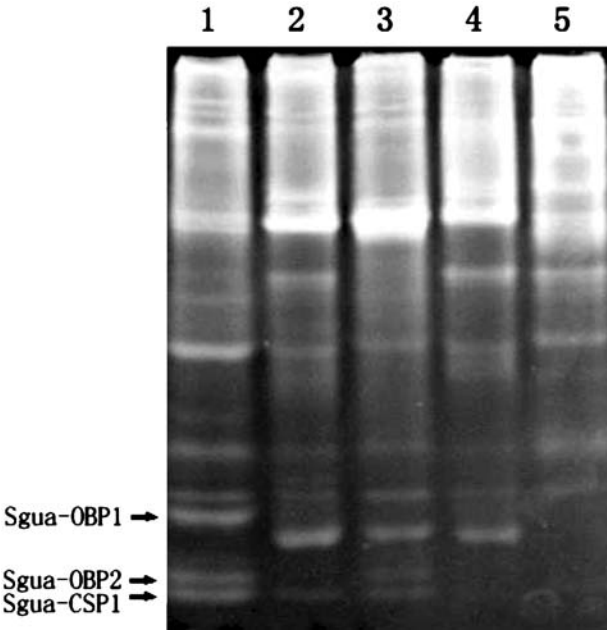


Fig. 1 Electrophoretic analysis under native conditions (15% native PAGE) of soluble antennal proteins from female *Scleroderma guani*. *lane 1*, female antenna; *lane 2*, female legs; *lane 3*, female head; *lane 4*, female thorax; and *lane 5*, female abdomen

its initial discovery. The nematode is likely native to North America (Dwinell 1997), but it has invaded both Asia and Europe (Mota et al. 1999; Kang et al. 2004).

Biological and ecological studies have indicated that the pine sawyer beetle, *Monochamus alternatus* Hope (Coleoptera: Cerambycidae), is the most important insect vector of *B. xylophilus* in China (Ding et al. 2001). Chen et al. (1996) first reported that the release of the parasitoid *Scleroderma guani* Xiao et Wu (Hymenoptera: Bethylidae) to control *M. alternatus* could be a highly effective management tactic for pine wilt disease.

The ant-like bethylid wasp *S. guani* is an ectoparasitoid of many woodboring insects. Since its discovery in Guangdong and Shandong provinces in 1973 and 1975 (Zhang 1980; Qin et al. 1982), respectively, it has been shown to occur in many provinces of China. Its hosts include more than 50 species of insects in 22 families from three orders (Chen and Cheng 2000).

Many studies have shown that *S. guani* could be an ideal candidate for biological control, especially in the suppression of longhorned beetles. Releases of *S. guani* have achieved effective and long-term control for *Anoplophora chinensis* (Chen 1995), *Anoplophora glabripennis* (Shi and Wang 2001), *Aromia bungii* (Jiang et al. 2001), *Mallambyx raddei* (Xie et al. 2000), *M. alternatus* (Xu et al. 2002; Wang et al. 2004), *Phytoecia rufiventris* (Cheng et al. 2003a), *Saperda populnea* (Pi et al. 2001; Feng et al. 2005), *Semanotus bifasciatus* (Li et al. 1993), and *Xylotrechus grayii* (Cheng et al. 2003b).

Odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) are two classes of small, water soluble, extracellular proteins that have been identified from insects in the sensillar lymph of chemosensilla. Their functions may be to solubilize and transport messenger molecules to the olfactory receptors (Kaissling 2001; Pelosi et al. 2005).

The OBPs, subdivided into pheromone binding proteins (PBPs) and general odorant binding proteins (GOBPs), have been identified and/or cloned from various insect orders, such as Lepidoptera (Nagnan-Le Meillour et al. 1996; Picimbon et al. 2001; Zhang et al. 2001; Picimbon and Gadenne 2002), Coleoptera (Peng and Leal 2001; Nagnan-Le Meillour et al. 2004), Dictyoptera (Riviere et al. 2003), Diptera (Koganezawa and Shimada 2002; Vogt et al. 2002), Hymenoptera (Calvello et al. 2003; Krieger and Ross 2005), Isoptera (Ishida et al. 2002a), Orthoptera (Ban et al. 2003a) and Hemiptera (Vogt et al. 1999) since first identified in 1981 (Vogt and Riddiford 1981). The OBPs are presumed to mediate the delivery of odorants to specific receptor proteins embedded in the dendritic membrane (Prestwich 1993). Steinbrecht (1998) proposed that OBPs function to: (1) solubilize and carry olfactory signal molecules; (2) filter signals at the periphery; (3) stimulate the olfactory system; (4) clean the peri-receptor space; and (5) deactivate odorants, but the physiological function of OBPs is still under investigation.

The CSPs (Angeli et al. 1999), variously named as OS-D-like proteins (McKenna et al. 1994), and sensory appendage proteins (Robertson et al. 1999), which have been identified in Phasmatodea (Marchese et al. 2000), Orthoptera (Ban et al. 2002; Ban et al. 2003b), Lepidoptera (Nagnan-Le Meillour et al. 2000; Picimbon et al. 2000), Hymenoptera (Calvello et al. 2003), Diptera (Wanner et al. 2004), Hemiptera (Jacobs et al. 2005), Coleoptera, Dictyoptera, Siphonaptera, and Phthiraptera (unpublished, but was indicated in the Pfam website: http://www.sanger.ac.uk/cgi-bin/Pfam/speciesdist.pl?acc=PF03392&id=OS-D&depth=all), have a wide tissue and anatomical distribution, including the head, antennae, proboscis, thorax, legs, sub-cuticular epithelium, pheromone glands, and ejaculatory duct (Wanner et al. 2004). CSPs share no sequence similarity with OBPs.

Although technology for mass rearing and releasing of *S. guani* has been developed, little is known of the behavioral chemical relationship between *S. guani* and *M. alternatus*.

Fig. 2 Analysis of Sgua-OBP1 protein. (**a**) Nucleotide and deduced amino acid sequences of Sgua-OBP1. The N-terminal sequences are in *bold type*; the putative signal peptide is in *italics*, the start ATG and stop TAA codons are in *bold italics*. The positions of sense (3′RACE) and anti-sense (5′RACE) oligonucleotides used for PCR are indicated. The conserved cysteine residues are *highlighted with gray background*. (**b**) Deduced amino acid sequences of Sgua-OBP1 aligned with seven of its most homologous proteins identified using NCBI BLASTP from other insect species. The conserved cysteine residues are *highlighted with gray background*. (**c**) Neighbor-joining tree (condensed tree, cut-off value is 50%) of sequences similar to Sgua-OBP1. Sequences used in alignment were indicated with species abbreviations followed with taxon identification and accession number. Species abbreviations are: Aaeg: *Aedes aegypti*, Acer: *Apis cerana cerana*, Acup: *Anomala cuprea*, Agam: *Anopheles gambiae*, Amel: *Apis mellifera*, Aoct: *Anomala octiescostata*, Aoct: *Anomala octiescostata*, Aosa: *Anomala osakana*, Aper: *Antheraea pernyi*, Bmor: *Bombyx mori*, Cflo: *Copidosoma floridanum*, Eori: *Exomala orientalis*, Hpic: *Heptophylla picea*, Hvir: *Heliothis virescens*, Llin: *Lygus lineolaris*, Mdom: *Musca domestica*, Msex: *Manduca sexta*, Pdiv: *Phyllopertha diversa*, Pjap: *Popillia japonica*, Pxyl: *Plutella xylostella*, Rpal: *Rhynchophorus palmarum*, Sfru: *Spodoptera frugiperda*, Sgua: *Scleroderma guani*, and Tcas: *Tribolium castaneum*

As a first step toward this understanding, we identified and cloned two odorant-binding proteins (OBPs) and one chemosensory protein (CSP) from female *S. guani*.

Methods and Materials

Insects Scleroderma guani were mass reared in the laboratory by using the yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), as a host. The larvae were reared at 28°C and 70% RH; no light was needed. The adults were stored at 10°C after emergence until dissected. Only live insects were dissected.

Sample Preparation and Protein Extraction Live insects were anesthetized on ice. Female antennae and control tissues (head, thorax, abdomen, and legs) were dissected under the microscope by using forceps and immediately transferred into 1.5 ml Eppendorf tubes that were immersed in liquid nitrogen. Tissues were then stored at −70°C until used. For protein extraction, samples were homogenized in an ice-cold glass homogenizer with 20 mM Tris–HCl, pH 7.4, and then centrifuged twice at 12,500×*g* for 10 min at 4°C. The supernatants were evaporated under vacuum (EZ550Q, Ultralow Freezer System, FTS Systems Inc., Stone Ridge, NY, USA). Protein was quantified by the method of Bradford (1976). For each set of experiments, approximately 4,000 antennae, 100 legs, 40 heads, 20 thoraces, and 10 abdomens were analyzed by nondenaturing polyacrylamide gel electrophoresis [15% native-polyacrylamide gel electrophoresis (PAGE)]. The amounts of soluble protein loaded in each lane were approximately 55 μg for antennal extracts and 70 μg each for leg, head, thoracic, and abdominal extracts.

Electrophoresis and Protein Sequencing PAGE was carried out at 100 V (constant voltage) at 4°C. Gels were stained by 0.1% Coomassie brilliant blue R250 in 50% (*v*/*v*) methanol and 10% (*v*/*v*) glacial acetic acid with constant shaking for 2–3 hr and then rinsed with destaining solution (10% methanol, 10% glacial acetic acid). For N-terminal sequencing, proteins were analyzed as above, then electroblotted onto PVDF membranes (0.2 μm, Millipore, Billerica, MA,USA) for 1.5 hr at 40 mA (constant current) and 4°C. Membranes were stained with 0.1% Coomassie brilliant blue R250 in 50% (*v*/*v*) methanol, 7% (*v*/*v*) acetic acid. Bands of interest were carefully excised and processed for sequencing by

a

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                                                                M  K  A  I  I
GATTCCAGTTGCATTGTATCTTCAACATTGATACGGGATTAAAGTTTCACAGAAGATACAATGAAAGCCATAATA

 L  V  V  A  L  C  S  I  Y  G  V  T  A  L  S  E  A  D  V  A  E  L  M  K  Y
TTAGTGGTTGCCCTCTGTTCCATCTATGGTGTTACGGCACTTTCGGAAGCAGACGTAGCGGAATTAATGAAATAT
                                        Sgua-OBP1-S(1-4)
 Q  D  A  C  I  A  E  S  G  V  D  P  V  L  I  E  N  A  K  K  G  D  V  A  P
CAAGATGCTTGCATCGCCGAATCCGGAGTTGATCCTGTTTTAATCGAAAACGCTAAGAAAGGTGACGTTGCCCCT

 D  E  N  L  A  C  F  A  S  C  M  L  Q  K  L  G  M  M  N  D  Q  G  V  L  N
GATGAGAATCTCGCTTGCTTTGCTTCTTGTATGTTGCAGAAACTTGGTATGATGAACGACCAAGGTGTCTTGAAC
                                                 Sgua-OBP1-AS1
 L  D  N  I  R  A  K  I  P  D  N  V  D  K  A  K  A  E  E  V  I  N  K  C  K
TTGGATAACATTCGCGCCAAGATTCCTGATAATGTGGATAAAGCAAAAGCAGAGGAAGTCATTAACAAATGCAAA

 D  V  P  G  N  H  H  C  L  K  A  G  N  F  V  Q  C  F  M  Q  H  K  E  F  A
GACGTTCCCGGAAACCATCACTGTTTGAAGGCAGGCAACTTCGTACAATGTTTCATGCAGCACAAAGAATTCGCT

 V  L  Q  *
GTTCTTCAGTAAGACTTAAACAGGATCTATAGAAATATCATGCTTTTGCATAATCCTGAGAAATCAGATTGATAG

GTTATTGATCGGTATGTCATATTCCATGCACTAGCGTACATGTACATAATGGTATCTCAACAACTGTGAATCAAT

AAAGAGCACTAAACAATGAAAAAAAAAAAAAAA
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b **Sgua-OBP1 Alignment**

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Sgua-OBP1       -------MKAIILVVALCSIYGVTA-LSEADVAELMKYQDACIAESGVDP-VLIENAKKG 51
Amel-OBP13      -------MKTIIFIFAFC-LVGILA-VSEESINKLRKIESVCAEENGIDL-KKADDVKKG 50
Llin-LAP        -------MRILVLFTAALTCVMAGE-LPEEMREMAQGLHDGCVEETGVDN-GLIGPCAKG 51
Aper-ABPX       ------DKLLAFIVTAGLFIFTVAS-LDGEMAELAKMIRDNCVDEIGVDV-TLLEQVDAG 52
Tcas-CG8462-PA  -------MKTVAVLLFLALAACTKQ-EDDDRQETIRQYRDDCIAETKVDP-ALIDRADNG 51
Aaeg-OBP56e     -------MKTIAAIVSFALIVGCMA-VTEEQKEAARQLAGKCMQQTGTSE-ESVQRLRNG 51
Cflo-OBP1       ----MKHFAAVVLFVAVCFVGAFSESLSNEEAEKLMEYKESCTAETGVDEAVLMQPYDDK 56
Aaeg-OBPRP      MTSFRLANLTVFLVLLFCFMRGVHSADDLSKIPEIKGYELHCIEASGITESSAKKLRNGD 60
                                  .                     *

Sgua-OBP1       DVAP-DENLACFASCMLQKLGMMNDQGVLNLDNIRAKIPDNVDKAKAEEVINKCKDVPGN 110
Amel-OBP13      IFDKNDEKLACYVDCMLKKVGFVNADTTFNEEKFRERT-TKLDSEQVNRLVNNCKDITES 109
Llin-LAP        -NFADDQKLKCYFKCVFGNLGVISDEGELDAEAFGSILPDNMQ-ELLP-TIRGCAGTTGA 108
Aper-ABPX       ANLMPDEKFKCYLKCTLETAGMMSD-GVVDIEIVLELLPEDLK-TKNENLLRKCDTQKGS 110
Tcas-CG8462-PA  -DFTDDAKLQCFSKCFYQKAGFVSETGDLLFDVIKDKIPKEANREKALAIIDKCKELKGA 110
Aaeg-OBP56e     DTSGADDNTKCFVQCFFQGAGVVDGEGNMQEAFVTEKLASEYGQAKAEEVVQRCRNNSGA 111
Cflo-OBP1       EELVQDEKLNCYFACILKKMDMMDSDGTINMETARSQLLRDLCPKKIDESVE-CLSQVGD 115
Aaeg-OBPRP      DIASPDQSIKCYVQCFFSKLRLMNEKGVVQKDKVLSLLGKLMEEDKAKKLAEKCDLRR-T 119
                     *.  *:  *     .:.    .                       *

Sgua-OBP1       HHCLKAGNFVQCFMQHKEFAVLQ-- 133
Amel-OBP13      NSCKKSSKLLQCFIDNNLMKIFE-- 132
Llin-LAP        DPCELAMNFNKCLQKVDPVNFMVI- 132
Aper-ABPX       DDCDTAFLTQVCWQNGNKADYFLI- 134
Tcas-CG8462-PA  DSCETVYLVHKCYFLHSYGTDKKTE 135
Aaeg-OBP56e     NACERSFSLLQCYIANRASLM---- 132
Cflo-OBP1       SPCNTAGKIFGCIMKIRTTCHDN-- 138
Aaeg-OBPRP      NPCDTAYAMYDCYRQNKAKLL---- 140
                  *       *
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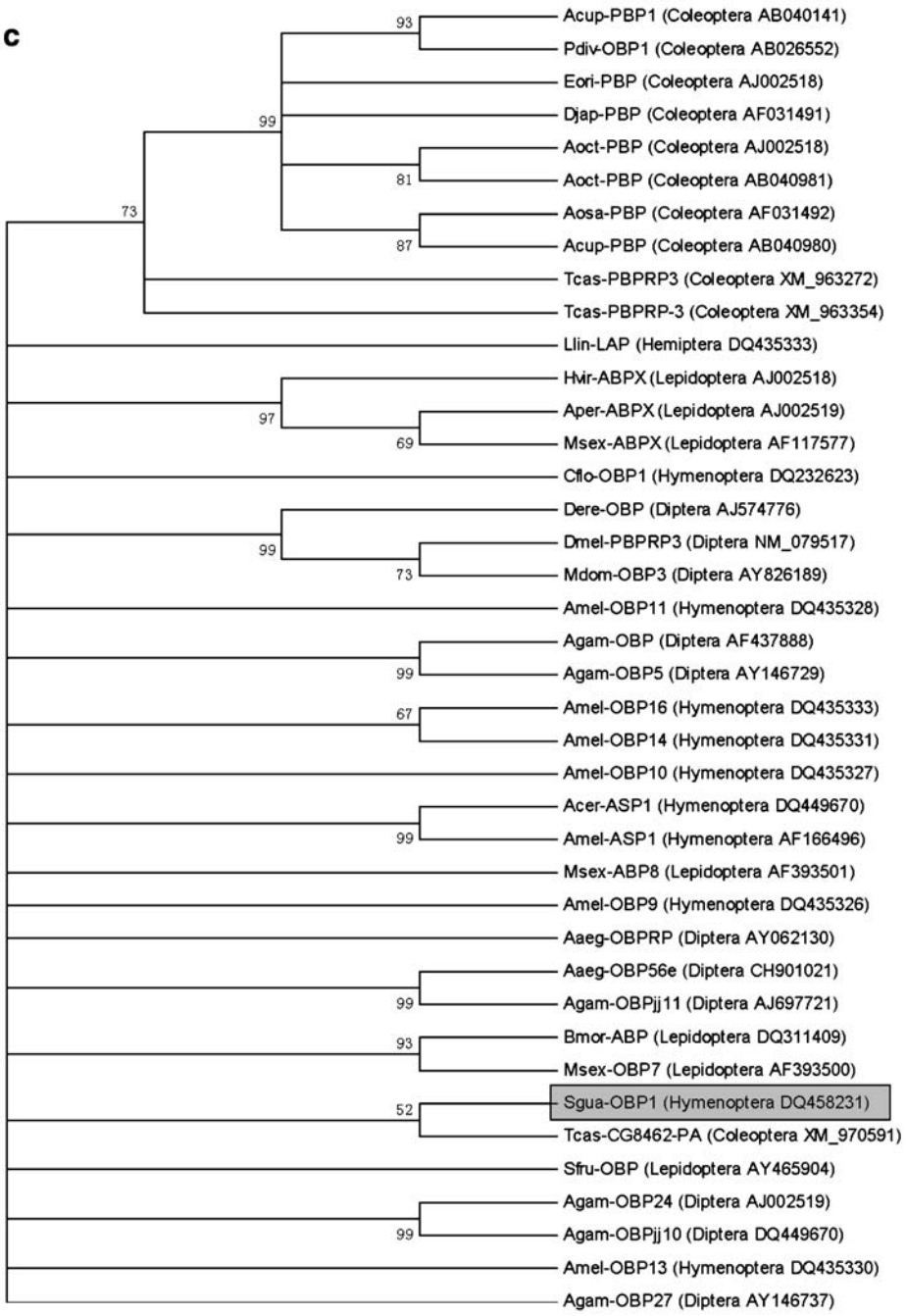


Fig. 2 (continued)

Edman degradation (the Procise® cLC Protein Sequencing System, Applied Biosystems, Shanghai GeneCore BioTechnologies Co., Ltd., Shanghai, China).

RNA Extraction, cDNA Synthesis, and 3′ and 5′ Rapid Amplification of cDNA Ends PCR Total RNA was extracted from 150 female antennae with Tri-Reagent (MRC, Cincinnati, OH, USA) according to the recommended protocol. The first strand cDNA was synthesized by using 3′-full rapid amplification of cDNA ends (RACE) core set (Takara, Dalian, China) according to the manufacturer's protocol. The reaction mixture contained 10 mM $MgCl_2$, deoxyribonucleotide triphosphate (diethylnitrophenyl thiophosphate (dNTP)) mixture (1 mM each), 0.25 U/μl AMV Reverse Transcriptase XL, 1 U/μl RNase inhibitor, 0.125 μM Oligo dT-3 site Adaptor Primer (Takara), template RNA, and sterile water, to a final volume of 20 μl. Polymerase chain reaction (PCR) with the reaction mixture for cDNA synthesis was performed as follows: 30°C (10 min), 50°C (30 min), 95°C (5 min), and 5°C (5 min) (Biometra Tgradient PCR thermocycler, Biometra GmbH i. L., Göttingen, Germany).

3′RACE-PCR Approximately 1 ng of cDNA from female antennae was used for 3′RACE-PCR. Degenerate sense primers were designed on the basis of the protein N-terminal sequences (Table 1) and synthesized by Bio Basic Inc. (Shanghai, China). These primers were used in a pair with an antisense Adapter Primer (Table 1) according to Oligo dT-3 site Adapter Primer in the 3′full RACE core set (Takara). PCR was performed with the following reaction mixture: 1×Ex Taq Buffer (Mg^{2+} free), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.5 μM sense primer, 0.2 μM antisense primer, 0.05 U/μl TaKaRa Ex Taq™ Hot Start Version (Takara) in 100 μl reaction volume. Reactions were performed on a Biometra Tgradient PCR thermocycler: 94°C (8 min); 5 cycles of 94°C (30 sec), 37°C (1 min), 2 min ramp, 72°C (1 min); followed by 30 cycles of 94°C (30 sec), 43°C (1 min), 2 min ramp, 72°C (1 min); and, finally, 72°C (10 min). The amplified cDNAs were purified after agarose electrophoresis by using TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (Takara) and ligated into a pGM-T Vector by using TOP10 Competent Cells (Tiangen Biotech Co., Ltd., Beijing, China). After transformation, positive clones were analyzed by PCR using vector specific primers. Clones of interest were amplified and plasmid DNAs were purified and sequenced in both directions with an ABI 3730 DNA Analyzer (Applied Biosystems, BGI LifeTech Co., Ltd., Beijing, China). Three independent clones for each of the proteins were sequenced to detect possible PCR mutations.

5′RACE-PCR 5′ cDNA sequences were obtained by using FirstChoice® RLM-RACE Kit (Ambion, Foster City, CA, USA) following the recommended protocol. Gene-specific antisense primers were designed from the results of the 3′RACE procedure (Table 1). These primers were used in a pair with a 5′ RACE Outer Primer (FirstChoice® RLM-RACE Kit) as the sense primer (Table 1). Reaction conditions for PCR were: 94°C (4 min); 30 cycles of 94°C (30 sec), 60°C (45 sec), 72°C (45 sec); and finally, 72°C (10 min). The Purified PCR products were ligated into pGM-T and positive clones were sequenced as described above.

Results

Protein Identification and Cloning Crude protein extracts from female antennae were analyzed by native PAGE. To better understand any differences in relative abundances of the target proteins in the various tissues, a greater amount of total protein was loaded for the leg, head, thoracic, and abdominal extracts. Hypothetically, we expected the target proteins

to be more abundant in the antennal extracts, so we hoped to increase the probability of visualizing them in the other tissue extracts if they were present. Coomassie blue staining revealed three bands in female antennal extracts that were tentatively identified as OBPs or CSPs (Fig. 1). These three bands represented highly mobile (i.e., likely small molecular weight proteins). One of them was antennal specific, whereas the other two bands suggested higher abundances in antennal extracts than in those from other tissues. The bands of interest were cut out after electroblotting onto PVDF membranes (0.2 μm, Millipore), and N-terminal sequenced: LSEADVAE (Sgua-OBP1), ADNDDPFESTRFK (Sgua-OBP2), and QESTPEYYTGKWN (Sgua-CSP1).

Full length sequences were obtained from 3′ and 5′ RACE-PCR, and all three were sent to GenBank for BLAST analysis. Two clones coded for members of OBP family; one coded for a member of the CSP family. They were named *Sgua-OBP1*, *Sgua-OBP2*, and *Sgua-CSP1*, respectively, and deposited in GenBank under the accession numbers DQ458231 (Sgua-OBP1 cDNA); DQ458232 (Sgua-OBP2 cDNA), DQ458233 (Sgua-CSP1 cDNA).

Characterization of Sgua-OBP1 The open reading frame (ORF) of Sgua-OBP1 encodes a 133 amino acid protein (Fig. 2a). Application of SignalP 3.0 http://www.cbs.dtu.dk/services/SignalP/) predicted that Sgua-OBP1 has an 18-residue hydrophobic signal peptide and that the mature protein contains 115 amino acid residues. The predicted signal peptide sequence matches the N-terminal amino acid sequence of the isolated protein. The theoretical p*I* (isoelectric point) and Mw (molecular weight) of the mature protein were computed by Compute p*I*/Mw http://www.expasy.org/tools/pi_tool.html). The p*I* is 4.92 and the Mw is 12,606.50. BLAST database analysis on Sgua-OBP1 indicated significant identities with previously described OBPs from other insects. The highest identity was 39% with the protein of Tcas-CG8462-PA (similar to CG8462-PA predicted by automated computational analysis, Coleoptera: *Tribolium castaneum*, Accession number: XM_970591); the next highest identity was 31% with the protein of Amel-OBP13 (Hymenoptera: *Apis mellifera*, Accession number: DQ435330). The alignment of Sgua-OBP1 and its homologs (Fig. 2b) was conducted in ClustalX (Thompson et al. 1997). The neighbor-joining tree for Sgua-OBP1 (Fig. 2c) and for Sgua-OBP2 and Sgua-CSP1 (see below, Figs. 3c and 4c) were constructed using MEGA version 3.1 (Kumar et al. 2004).

Characterization of Sgua-OBP2 The ORF of Sgua-OBP2 encodes a 142-amino acid protein (Fig. 3a). SignalP predicted that Sgua-OBP2 has a 20 residue hydrophobic signal peptide and

Fig. 3 Analysis of Sgua-OBP2 protein. (**a**) Nucleotide and deduced amino acid sequences of Sgua-OBP2. The N-terminal sequences are in *bold type*; the putative signal peptide is in *italics*, the start ATG and stop TGA codons are in *bold italics*. The positions of sense (3′RACE) and anti-sense (5′RACE) oligonucleotides used for PCR are indicated. The conserved cysteine residues are *highlighted with gray background*. (**b**) Deduced amino acid sequences of Sgua-OBP2 aligned with seven of its most homologous proteins identified using NCBI BLASTP from other insect species. The conserved cysteine residues are *highlighted with gray background*. (**c**) Neighbor-joining tree (condensed tree, cut-off value is 50%) of sequences similar to Sgua-OBP2. Sequences used in alignment were indicated with species abbreviations followed with taxon identification and accession number. Species abbreviations are Aaeg: *Aedes aegypti*, Acer: *Apis cerana cerana*, Acup: *Anomala cuprea*, Agam: *Anopheles gambiae*, Amel: *Apis mellifera*, Aoct: *Anomala octiescostata*, Aosa: *Anomala osakana*, Aper: *Antheraea pernyi*, Cqui: *Culex pipiens quinquefasciatus*, Eori: *Exomala orientalis*, Hpic: *Heptophylla picea*, Hvir: *Heliothis virescens*, Msex: *Manduca sexta*, Pdiv: *Phyllopertha diversa*, Pjap: *Popillia japonica*, Rpal: *Rhynchophorus palmarum*, Sexi: *Spodoptera exigua*, Sfru: *Spodoptera frugiperda*, Sgua: *Scleroderma guani*, Slit: *Spodoptera litura*, Snon: *Sesamia nonagrioides*, Tcas: *Tribolium castaneum*, and Znev: *Zootermopsis nevadensis*

a

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                                                                M  K  S  F  S  A  L  V  V  L  L  I
GCGATTGCATCGCATAAGTTTACTCACGACGAACTCAGCATGAAGAGCTTTTCCGCACTCGTTGTTCTTCTCATT

 A  I  L  A  S  A  L  A  A  D  N  D  D  P  F  E  S  T  R  F  K  C  Q  K  E
GCTATTCTTGCGTCTGCTTTAGCAGCGGATAACGACGATCCCTTCGAATCGACCAGATTCAAATGTCAAAAGGAA
                          Sgua-OBP2-S1
 Y  G  F  S  D  E  E  L  L  A  G  E  E  N  L  E  P  M  K  C  F  L  F  C  F
TACGGCTTCTCCGACGAGGAACTACTTGCCGGGGAGGAAAATCTTGAGCCTATGAAATGTTTCTTGTTTTGCTTC

 L  K  D  L  Q  I  A  D  D  T  G  N  F  D  P  A  V  A  T  M  M  L  D  D  D
TTGAAAGATCTACAAATTGCGGATGATACAGGTAACTTTGATCCAGCTGTAGCTACAATGATGCTTGACGATGAC

 I  R  D  T  A  K  S  A  I  Y  K  C  H  A  D  Y  L  T  V  S  E  P  C  Q  H
ATACGCGATACCGCCAAGTCAGCTATTTACAAATGCCACGCAGATTATTTAACAGTCAGTGAGCCGTGCCAGCAT
                                                    Sgua-OBP2-AS1
 S  Y  E  V  V  K  C  F  K  E  T  L  P  E  I  Y  K  M  L  G  I  F  R  P  P
TCCTATGAGGTCGTAAAATGTTTCAAGGAGACGTTGCCAGAGATCTACAAGATGCTCGGAATATTCCGTCCGCCC

 Q  I  M  S  Q  *
CAGATCATGTCACAGTGATGCAATCAGTATATGTATGTGCTTTAGCAATGAATGTAATAATTGTTATTAGAAATA

AATAATAAATTCAAAAAAAAAAAAAAA
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b

Sgua-OBP2 Alignment

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Pdiv-OBP1   ----------------------------EEMEELAKQLHNDCVGQTGVDEAHITTVK 29
Acup-PBP1   ----------------------------MSEEMEELAKQLHNDCVGQTGVDEAHITTVK 31
Msex-ABP3   -----MITATLHVVFALLGFVYGAKNKPVFSEEIKEIIQTVHDECVGKTGVSEEDIANCE 55
Aaeg-OBP    -MIRFIVFVSSCLVAVSIADVTPRRDAEYPPPELLQALKPLRDICQKKTGVSDEAILEFS 59
Amel-ASP1   MVSNTKQAFIYSLALLCLHAIFVNAAPDWVPPEVFDLVAEDKARCMSEHGTTQAQIDDVD 60
Amel-OBP9   ---MFKNYHFFFILVITLIFLYFG-------------EADIKKDCRKESKVSWAALKKMK 44
Sgua-OBP2   ----MKSFSALVVLLIAILASALAADND-------DPFESTRFKCQKEYGFSDEELLAGE 49
Znev-OBP1   -----MKTSERVLASAILLLLGVADLASGLTGRAFERAKEVDEKCRSENNVERAYFEKFI 55
                                                  *  :       :

Pdiv-OBP1   DQKG--FPDDEKFKCYLKCLMTEMAIVGDDGIVDIEAAVGVLPDELKAKAEPVMRKCGFK 87
Acup-PBP1   DQKG--FPDDEKFKCYLKCLMTEMAIVGDDGIVDIEAAVGVLPDELKAKAEPVMRKCGFK 89
Msex-ABP3   NGI---FKEDVKLKCYMFCLLEVAGLADEDGTVDYDMLVSLIPEEYSERASKMIFACNHL 112
Aaeg-OBP    DGK---VHEDEKLKCYMNCLFHEAKVVDDTGHVHLEKLHDALPDSMRDIAMHMGKRCLYP 116
Amel-ASP1   KGN---LVNEPSITCYMYCLLEAFSLVDDEANVDEDIMLGLLPDQLQERAQSVMGKCLPT 117
Amel-OBP9   AGDM--EQDDQNLKCYLKCFMTKHGILDKNAEVDVQKALRHLPRSMQDSTKKLFNKCKSI 102
Sgua-OBP2   ENL-------EPMKCFLFCFLKDLQIADDTGNFDPAVATMMLDDDIRDTAKSAIYKCHAD 102
Znev-OBP1   KARIDEIDPPDNYKCFVKCVMVELMALNDEGDFNVDEELQNVPPEIVEEGHRIVKTCHGT 115
                       .*:: *.:      .. . ..       :  .           *

Pdiv-OBP1   PG--ANPCDNVYQTHKCYFETDPQSYMIV---------- 114
Acup-PBP1   PG--ANPCDNVYQTHKCYFETDPHSYMIV---------- 116
Msex-ABP3   DTPEKDKCQRSFDVHKCTYEKDPEFYFLF---------- 141
Aaeg-OBP    EG--ENLCEKAFWLHKCWKESDPKHYFLI---------- 143
Amel-ASP1   SG--SDNCNKIYNLAKCVQESAPDVWFVI---------- 144
Amel-OBP9   QN--EDPCEKAYQLVKCYVEFHPEVLQTVPFL-------- 132
Sgua-OBP2   YLTVSEPCQHSYEVVKCFKETLPEIYKMLGIFRPPQIMSQ 142
Znev-OBP1   PG--KDPCDKAYQVHKCYHKENPELYSLILHYWENASQ-- 151
                 : *:. :   **  :  *.    .
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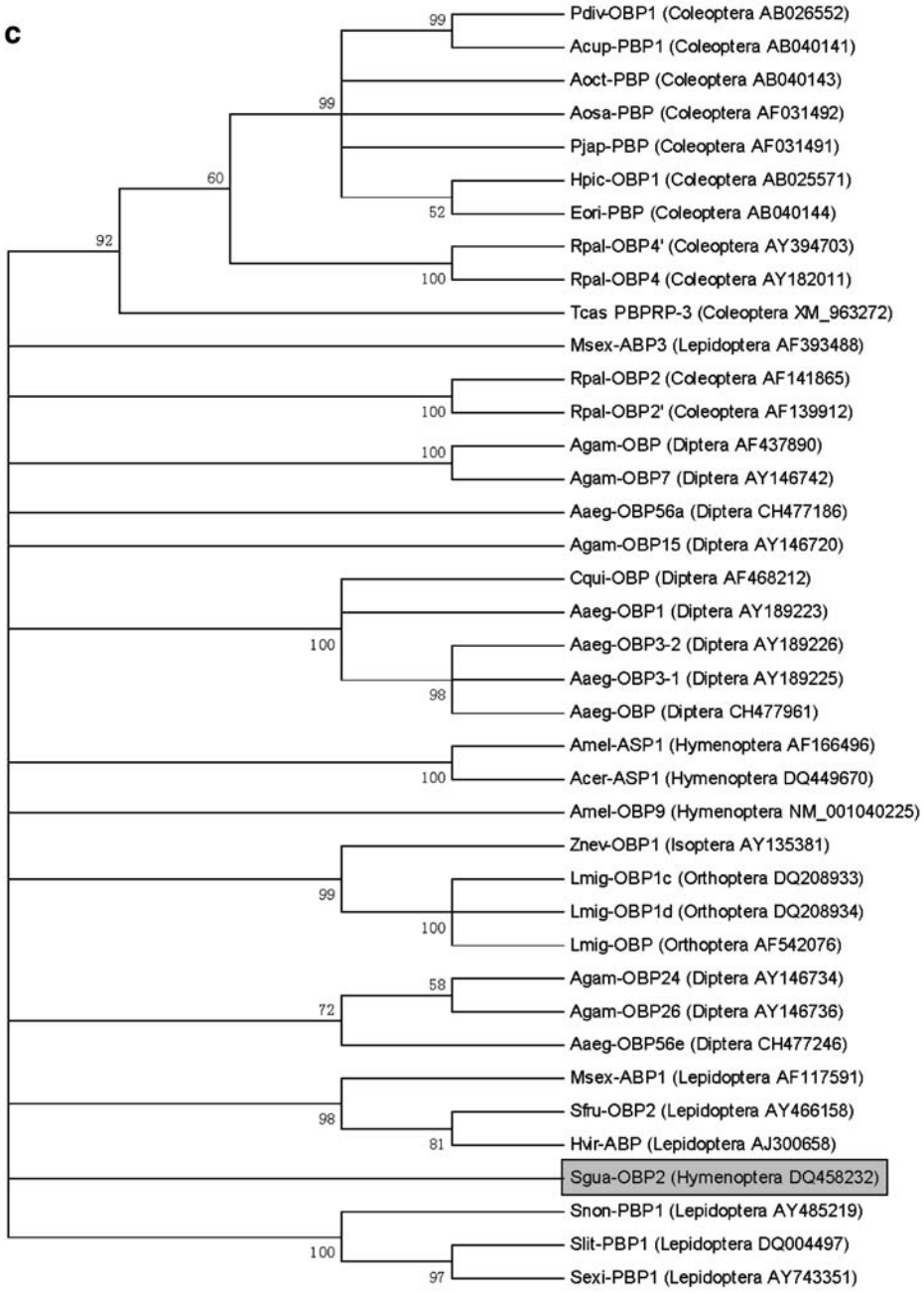


Fig. 3 (continued)

that the mature protein contains 122 amino acid residues. The predicted signal peptide sequence matches the N-terminal amino acid sequence of the isolated protein. The theoretical p*I* (isoelectric point) and Mw (molecular weight) of the mature protein are 4.39 and 14,041.91, respectively. BLASTP analysis also indicated that Sgua-OBP2 is homologous to insect OBPs, but the identities are quite low. The highest identity was less than 30% (Pdiv-OBP1, Coleoptera: *Phyllopertha diversa*; Accession number: AB026552) (Fig. 3b). The alignment between Sgua-OBP1 and Sgua-OBP2 by using matrix of BLOSUM62 (Henikoff and Henikoff 1992) showed no significant similarity (data not shown).

Characterization of Sgua-CSP1 The ORF of Sgua-CSP1 encodes a 129-amino acid protein (Fig. 4a). SignalP predicted a 20-residue hydrophobic signal peptide and that the mature protein contains 109 amino acid residues. The predicted signal peptide sequence matches the N-terminal amino acid sequence of the isolated protein. The theoretical p*I* (isoelectric point) and Mw (molecular weight) of the mature protein are 5.95 and 13,096.95, respectively. BLASTP analysis indicated that Sgua-CSP1 is homologous to other insect CSPs (Fig. 4b). The highest identity with another CSP is 36% (Vcra-CSP, Hymenoptera: *Vespa crabro*, Accession number: AY803290).

Discussion

Generally, OBPs are considered to be antennal-specific, whereas CSPs are widely distributed throughout the body, including the head, antennae, proboscis, thorax, legs, sub-cuticular epithelium, pheromone glands, and ejaculatory duct (Wanner et al. 2004). However, an antennal-specific CSP has been reported in the Argentine ant (Ishida et al. 2002b). Calvello et al. (2003, 2005) also reported an antennal-specific CSP and an OBP expressed in legs and wings in the wasps, *Polistes dominulus* and *Vespa crabro*. In our work, three proteins originally isolated from the female antennae of *S. guani* were expressed differently. The protein Sgua-OBP1 was antennal specific in females; Sgua-OBP2 was present in the antennae and head; and Sgua-CSP1 was distributed broadly throughout the whole insect body. However, native PAGE analysis (Fig. 1) indicated that both Sgua-OBP1 and Sgua-CSP1 were more abundant in antennal extracts than in extracts from any of the other body tissues that we analyzed. In pilot studies (data not shown), extracted protein was loaded for native PAGE by allocating the same quantities (approximately 55 μg) to each lane. The resulting gel showed very weak bands corresponding to Sgua-OBP2 in head and to Sgua-CSP1 in legs, head, thorax and abdomen, respectively. Further research by using immunochemical methods will be necessary to demonstrate more fine structure anatomical expression for these three proteins.

OBPs are usually expressed in sensillar support cells and secreted by these cells into the aqueous sensillar lumen. The proteins have ranged from 120 to 150 amino acids in length and have a mass of approximately 14 kDa. OBPs contain six cysteine residues that form disulfide bonds (a hallmark of OBPs) to stabilize the functionally relevant three-dimensional structure of the protein (Leal et al. 1999; Briand et al. 2001; Horst et al. 2001a, b). CSPs contain 100 to 110 amino acid residues, but have four conserved cysteine residues and six á-helices (Campanacci et al. 2001a, b, 2003). They share no sequence similarity to OBPs. The mature proteins of Sgua-OBP1, Sgua-OBP2, and Sgua-CSP1 are 115, 122, and 109 amino acids in length, and 12, 14, and 13 kDa in mass, respectively. The

Fig. 4 Analysis of Sgua-CSP1 protein. (**a**) Nucleotide and deduced amino acid sequences of Sgua-CSP1. The N-terminal sequences are in *bold type*; the putative signal peptide is in *italics*, the start ATG and stop TAA codons are in *bold italics*. The positions of sense (3′RACE) and anti-sense (5′RACE) oligonucleotides used for PCR are indicated. The conserved cysteine residues are *highlighted with gray background*. (**b**) Deduced amino acid sequences of Sgua-CSP1 aligned with its most homologous protein identified using NCBI BLASTP from other insect species. The conserved cysteine residues are *highlighted with gray background*. (**c**) Neighbor-joining tree (condensed tree, cut-off value is 50%) of sequences similar to Sgua-CSP1 Sequences used in alignment were indicated with species abbreviations followed with taxon identification and accession number. Species abbreviations are Acer: *Apis cerana cerana*, Agam: *Anopheles gambiae*, Amel: *Apis mellifera*, Bmor: *Bombyx mori*, Cfum: *Choristoneura fumiferana*, Cson: *Culicoides sonorensis*, Dmel: *Drosophila melanogaster*, Gori: *Gryllotalpa orientalis*, Hass: *Helicoverpa assulta*, Hvir: *Heliothis virescens*, Hzea: *Helicoverpa zea*, Lmad: *Leucophaea maderae*, Lmig: *Locusta migratoria*, Lobl: *Lonomia oblique*, Mdir: *Metopolophium dirhodum*, Mper: *Myzus persicae*, Msex: *Manduca sexta*, Mvic: *Megoura viciae*, Nrib: *Nasonovia ribis-nigri*, Sgre: *Schistocerca gregaria*, Sgua: *Scleroderma guani*, Slit: *Spodoptera litura*, Tcas: *Tribolium castaneum*, and Vcra: *Vespa crabro*

only point of divergence of our OBPs and CSP from the criteria listed above is that Sgua-CSP1 contains six cysteine residues, instead of the typical four. However, Sgua-CSP1 is not homologous to OBPs from other insects and shares high identity with previously described CSPs, so we have placed it in this group. Further research on the crystal structure and binding properties by NMR will help to clarify the function of this protein.

Database searches suggest that the Sgua-OBP1 and Sgua-OBP2 are homologs of the OBPs from other insects and that Sgua-CSP1 shares more identity with previously described CSPs. However, the identities are all quite low and the neighbor-joining tree analyses of sequences also show very low similarity with other proteins (Figs. 2c, 3c, and 4c). A search of the Protein Family Database http://www.sanger.ac.uk/Software/Pfam/) revealed that approximately 529 OBPs and 183 CSPs have been identified/cloned so far. There are 5 OBPs and 3 CSPs from Dictyoptera; 3 CSPs from Phasmatodea; 3 OBPs and 46 CSPs from Orthoptera; 142 OBPs and 57 CSPs from Lepidoptera; 32 OBPs and 3 CSPs from Coleoptera; 63 OBPs and 12 CSPs from Hymenoptera; 1 CSP from Siphonaptera; 281 OBPs and 31 CSPs from Diptera; 1 CSP from Phthiraptera; and 3 OBPs and 26 CSPs from Hemiptera. Sgua-OBP1, Sgua-OBP2, and Sgua-CSP1 are the first in these groups to be identified from an ectoparasitic wasp, which may explain why these three proteins have quite low identities with other known OBPs and CSPs, which have been isolated and identified primarily from phytophagous insects. Insect predators and parasitoids may locate the adult host by eavesdropping on the sex pheromones emitted by host or may locate the larval host by exploiting the "alarm signals" produced and released by injured plant.

Usually, the sex ratio in *S. guani* is biased in favor of females (17 to 1 female to male). Males only survive several days to copulate (Chen and Cheng 2000). In contrast, female *S. guani* survive much longer to find hosts. We hypothesize that OBPs and CSPs may be differentially expressed in males and females, but it is very difficult to collect males for investigation, so males were not included in the first part of this investigation. In the future, we plan to focus on the identification and cloning of olfactory proteins from male *S. guani*. This will help in determining the specificity of the proteins discussed in this paper and may help to explain their function. Further studies may also include monitoring the expression of the OBPs and CSPs during the life cycle.

Acknowledgments This work was supported by the China National Basic Research Program (973 Program) of Invasion Biology and Control Strategies for Alien Species in Agriculture and Forestry (2002CB111407). We also thank Dr. Patricia Nagnan-Le Meillour (INRA, Versailles, France) for her advice with this work.

a

```
                                                                M  K  S  I
ATACAGTCTTCAATATACTTTTGCTATTTCATTATAGTTTTCTTTGTGTGATCGAAATTTAAGATGAAGAGCATT

 I  V  V  F  T  V  F  A  I  V  F  A  Q  E  S  T  P  E  Y  Y  T  G  K  W  N
ATAGTTGTTTTCACTGTGTTCGCGATAGTATTCGCACAAGAATCGACACCTGAATATTACACTGGAAAGTGGAAT
                                                Sgua-CSP1-S1
 D  L  N  T  H  D  I  V  D  N  A  R  L  F  K  K  Y  K  Q  C  I  M  A  E  T
GATCTCAATACTCATGATATCGTCGATAATGCACGTCTTTTTAAGAAATACAAGCAGTGCATAATGGCTGAAACA

 N  T  G  C  P  Q  E  V  I  E  L  K  K  V  L  P  E  A  L  E  T  V  C  S  K
AATACGGGTTGTCCTCAGGAAGTTATAGAGCTGAAAAAAGTTCTACCCGAGGCATTAGAGACGGTATGTTCCAAG
                                                          Sgua-CSP1-AS1
 C  S  P  V  Q  V  E  K  I  R  D  T  L  K  Y  V  C  E  K  R  K  T  D  F  D
TGTTCTCCAGTTCAAGTTGAGAAAATTCGCGACACTCTCAAATATGTTTGCGAAAAGCGCAAAACTGATTTTGAT

 D  I  L  K  H  I  D  P  E  G  T  H  R  P  K  F  E  E  K  F  G  T  L  G  C
GATATCTTGAAGCATATTGATCCTGAAGGCACGCATCGTCCAAAATTCGAAGAGAAATTTGGAACTCTTGGATGT

 *
TAAGAAAGATGTCGAAACGTTAACGAAAAATATTAAATGTCTTATTCTTAATTAAAACAGTTTATTGATCTTCAA

TGTATACTATTGCAAATAGTAATTAAAGAGAAACGCAATTCTGTAAATAAACAAAAAAAAAAAAAAA
```

b

Sgua-CSP1 Alignment

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Mper-OS-D1      -MAHLNLFVVLVASLVCFTLAEEKYTTKFDNFDVDKVLNNNRILTSYIKCLLDE--GNCT 57
Nrib-OS-D1      -MAHLNLFVVLVASLVCFTLAEEKYTTKFDNFDVDKVLNNDRILTSYIKCLLEQ--GNCT 57
Tcas-CSP7       --MKLISAVILCAFLVAVSAAENKYTNKYDNVDVDKILNNDRVLTNYIKCLMDE--GPCT 56
Lmig-CSP        -------MQALTLVLFALVASAAAYTTKYDNIDLDEILNNERLLKKYHECLMSDSDASCT 53
Acer-ASP3       MKVSIICLVLMAAIVLVAARPDESYTSKFDDINVDEILHSDRLLNNYFKCLMDE--GRCT 58
Amel-CSP3       MKVSIICLVLMAAIVLVAARPDESYTSKFDNINVDEILHSDRLLNNYFKCLMDE--GRCT 58
Vcra-CSP        MKTTFIFLTVLAVIVVSLAQ-ESMYTTKFDNINVQEILHNDRLLNNYVKCLLDQ--GRCT 57
Sgua-CSP1       -MKSIIVVFTVFAIVFAQESTPEYYTGKWNDLNTHDIVDNARLFKKYKQCIMAETNTGCP 59
                          :   :.         ** *:::.: ..::.. *::..* :*:: :    *.

Mper-OS-D1      NEGRELRKVLPDALKTDCSKCTEVQKDRSEKVIKFLIKNRSTDFDRLTAKYDPSGEYKKK 117
Nrib-OS-D1      NEGRELKRVLPDALKTDCNKCTEVQKNRSGKVIKFLIKNRSNDFDRLIAKYDPTGEYKKK 117
Tcas-CSP7       SEGRELKKTLPDALSSGCTKCNQKQKETAEKVIRHLTQKRARDWERLSKKYDPQGQYKKR 116
Lmig-CSP        PDGKELKVSIPDALVTDCSKCNEKQKEGSNKVIRFLIQKKEDLWKPLQAKYDPEGTYLKK 113
Acer-ASP3       AEGNELKRVLPDALATDCKKCTDKQREVIKKVIKFLVENKPELWDSLANKYDPDKKYRVK 118
Amel-CSP3       AEGNELKRVLPDALATDCKKCTDKQREVIKKVIKFLVENKPELWDSLANKYDPDKKYRVK 118
Vcra-CSP        ADAIELKKSLPDALETECSKCSPKQKEFAEEAMKFLSHNKKDIWEKLLAKYDPEKKYRSK 117
Sgua-CSP1       QEVIELKKVLPEALETVCSKCSPVQVEKIRDTLKYVCEKRKTDFDDILKHIDPEGTHRPK 119
                 :  **:  :*:** : *.**.  * :   ..::.: .::   :. :  : **   :  :

Mper-OS-D1      IEKFDSEKAAAAKH 131
Nrib-OS-D1      IEKFDAERAAAAKH 131
Tcas-CSP7       YEEHVATSRAA--- 127
Lmig-CSP        HPELLSA------- 120
Acer-ASP3       FEEEAKKLGINV-- 130
Amel-CSP3       FEEEAKKLGINV-- 130
Vcra-CSP        FEDRAKEADIKI-- 129
Sgua-CSP1       FEEKFGTLGC---- 129
```

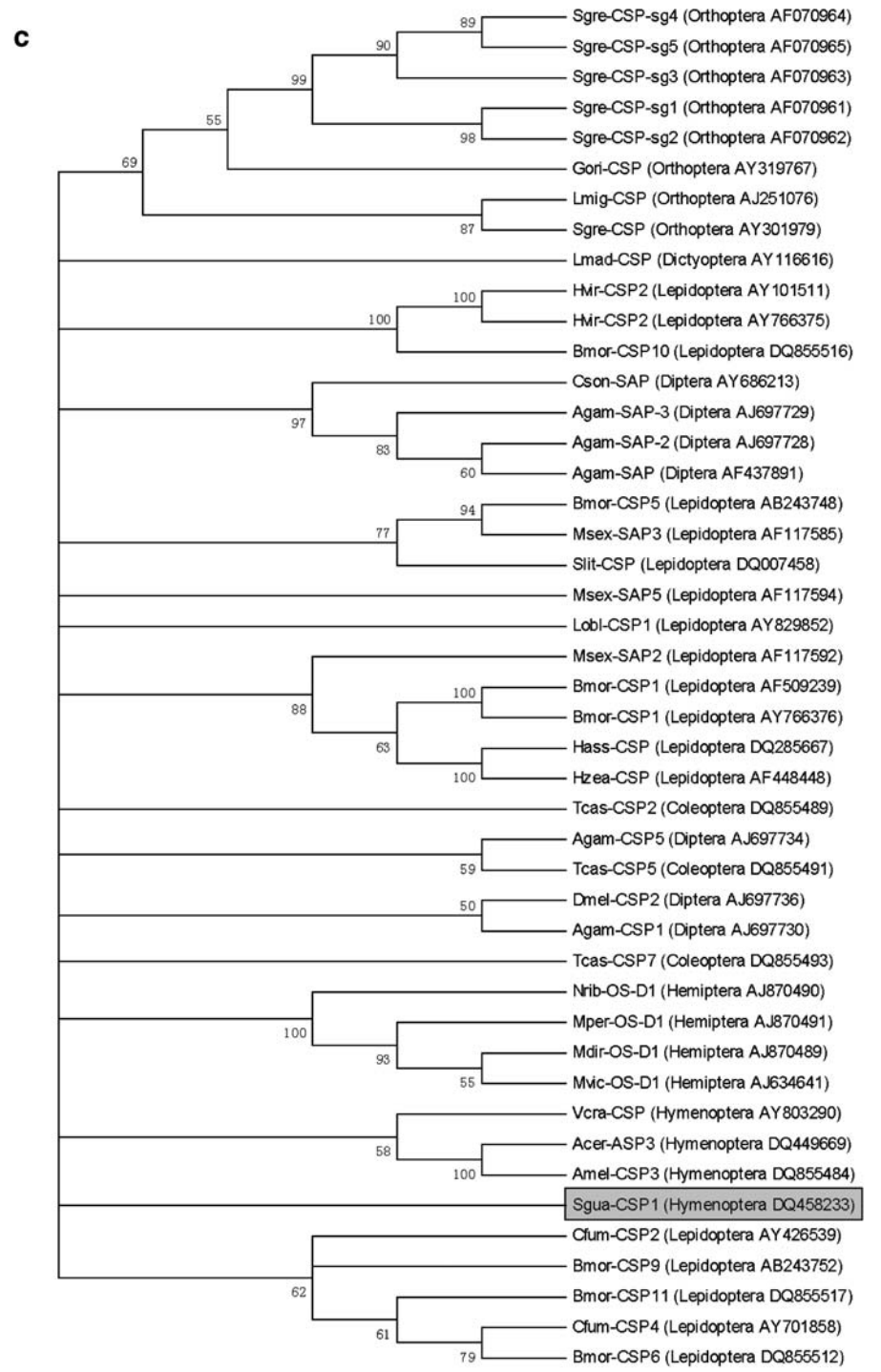


Fig. 4 (continued)

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these FAAs were not found in ten of the insects studied here, their identification in two additional orders of insects suggests that FAAs are more common than previously reported and may have physiological roles in a wide range of insects besides caterpillars.

Keywords Volicitin · Lepidoptera · *Drosophila* · Teleogryllus · Larvae · Elicitors · LCMS-IT-TOF · Fatty acid amides · Volicitin

Introduction

Volicitin and its related analogs, generically called fatty acid amides (FAAs), were first identified as elicitors that induce plant volatile emission in the context of plant–herbivore interactions (Alborn et al. 1997). The mechanism by which volicitin triggers the systemic release of volatiles by corn plants has been studied in detail (Truitt et al. 2004), but how and why plants have evolved such a complex chemical signaling response to compounds of herbivore origin continues to engage researchers. Equally intriguing from an evolutionary perspective, volicitin and other FAAs exist in the oral secretions of some caterpillar species despite the propensity of some FAAs to elicit volatile emission in plants, thus, ultimately attracting the caterpillar's natural enemies. Lait et al. (2003) reported a key enzyme involved with the biosynthesis of *N*-linolenoyl-L-glutamine, a structural analog of volicitin, in alimentary tissues of *Manduca sexta* larvae. Our recent study of volicitin synthesis by *Spodoptera litura* revealed that glutamine is selectively incorporated into FAAs even though other amino acids are also found in the hemolymph and lumen of the gut (Yoshinaga et al. 2005). This suggests that FAAs could be involved with glutamine metabolism, and we hypothesize that they likely play a fundamental role in the physiology of lepidopteran larvae by virtue of their occurrence in multiple species (Alborn et al. 1997, 2003; Pare et al. 1998; Pohnert et al. 1999; Halitschke et al. 2001; Mori et al. 2001, 2003). Thus far, the search for FAAs has been limited to a small number of caterpillar species. The objective of this study was to search for FAAs in orders of insects other than Lepidoptera. We performed liquid chromatography/mass spectrometry-ion trap-time-of-flight (LCMS-IT-TOF) analysis on extracts from five species of Orthoptera, three species each of Hemiptera and Hymenoptera, and one species each of Coleoptera and Diptera (Table 1).

Methods and Materials

Insect Material and Gut or Larval Extracts Laboratory strains of *Drosophila melanogaster* (Canton S, Oregon R) were reared on artificial diet Formula 4-24 (Carolina Biological Supply Co.) that was supplemented with α-linolenic acid (10 μl/6 g wet diet) to compensate for dietary deficiency. Final instars were removed from the diet, rinsed with distilled water, and blotted dry with a paper towel. Larvae (20 per sample) were boiled in a microcentrifuge tube for 20 min with 10 μl of distilled water to prevent FAA hydrolysis by endogenous enzyme(s). Whole larvae were homogenized in 50 μl of acetonitrile, containing 0.3 μg of *N*-palmitoleoyl-L-glutamine as an internal standard, and then centrifuged for 10 min at 13,000×*g* to obtain a supernatant for LCMS and LCMS-IT-TOF analysis. Adult *Teleogryllus taiwanemma* (laboratory strain) were fed a natural diet of gramineous weeds for 48 hr and then frozen. Other adult specimens of Orthoptera, Hemiptera, Hymenoptera, and Coleoptera were collected from the field and frozen immediately after taxonomic identification. Each of these insects was sliced longitudinally with a razor, and the frozen

Table 1 Insect species used for LCMS-IT-TOF screening and FAA composition of extracts from gut contents or whole larval body

Order	Family	Species	Food Plants	FAAs							
				(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Diptera	Drosophilidae	*Drosophila melanogaster*	Artificial diet	o	o	+	+	o	o	++	++
Orthoptera	Pyrgomophidae	*Atractomorpha lata*	*Eleusine indica*	−	−	−	−	−	−	−	−
	Acrididae	*Acrida cinera*	*Setaria viridis*	−	−	−	−	−	−	−	−
	Catantopidae	*Patanga japonica*	Unknown plants	−	−	−	−	−	−	−	−
	Gryllidae	*Teleogryllus taiwanemma*	*Eleusine indica*	+	o	+	+	+	o	++	++
		Teleogryllus emma	Unknown plants	+	o	+	+	+	o	++	++
Hemiptera	Pemtatomidae	*Eurydema rugosa*	*Brassica rapa* L.	−	−	−	−	−	−	−	−
	Coreidae	*Acanthocoris sordidus*	*Ipomoe batatas*	−	−	−	−	−	−	−	−
	Cicadellidae	*Bothrogonia ferruginea*	Unknown Plants	−	−	−	−	−	−	−	−
Coleoptera	Scarabaeidae	*Oxycetonia jucunda*	Unknown Plants	−	−	−	−	−	−	−	−
Hymenoptera	Argidae	*Arge* sp	*Rosa* sp.	−	−	−	−	−	−	−	−
	Tenthreddnidae	*Athalia rosae ruficornis*	*Brassica oleracea* L.	−	−	−	−	−	−	−	−
	Cimbicidae	*Zeraea akebii*	*Akebia* sp.	−	−	−	−	−	−	−	−

(1) volicitin, *(2)* *N*-hydroxylinoleoyl-L-glutamine, (*3*) *N*-hydroxylinolenoyl-L-glutamic acid, (*4*) *N*-hydroxylinoleoyl-L-glutamic acid, *(5)* *N*-linolenoyl-L-glutamine, *(6)* *N*-linoleoyl-L-glutamine, *(7)* *N*-linolenoyl-L-glutamic acid, *(8)* *N*-linoleoyl-L-glutamic acid, ++ major component (>30% of total component), + minor component (>3%), *o* trace component (< 3%), − not detected

midgut contents were transferred, by forceps, to microcentrifuge tubes. Midgut contents (approximately 30 μl) were boiled with 10 μl of distilled water, homogenized with 50 μl of acetonitrile, containing 0.3 μg of *N*-palmitoleoyl-L-glutamine, and centrifuged as described above to obtain a supernatant for LCMS and LCMS-IT-TOF analysis.

LCMS and LCMS-IT-TOF Analyses MS detection focused on ions at *m/z* 421, 422, 423, 424, 405, 406, 407, 408, 277, and 279, by using selected ion monitoring mode. Negative ESI mass spectral measurements were conducted with an LCMS-2010A instrument (Shimadzu, Kyoto) combined with an HPLC system (LC-10ADvp pump, CTO-10ACvp column oven, and SCL-10AVvp system controller; Shimadzu, Kyoto). A reverse-phase column (Mightysil RP-18 GP, 50×2.0 mm i.d.; Kanto Chemical Co., Inc., Tokyo) was eluted (0.25 ml min^{-1}) with a gradient of 20–95% acetonitrile, containing 0.08% acetic acid, in water containing 0.05% acetic acid, over 15 min with column temperature maintained at 40°C. Sample solutions containing FAAs were further analyzed by using a Prominence HPLC system coupled to LCMS-IT-TOF (Shimadzu, Kyoto). A Cosmosil 5C_{18}-AR-II column (50×2.0 mm i.d.; Nakalai Tesque, Kyoto) was eluted (0.2 ml min^{-1}) with a gradient of 20–90% acetonitrile, containing 0.08% acetic acid, in water containing 0.05% acetic acid, over 15 min with column temperature maintained at 40°C. The MS was

operated with probe voltage of 4.50 kV, CDL temperature of 200°C, block heater temperature of 200°C, nebulizer gas flow of 1.5 L min^{-1}, ion accumulation time of 10 msec, MS range of *m/z* 200 to 500, MS^2 range of *m/z* 100 to 500, CID parameters as follows: energy, 80%; collision gas, 100%. The precursor ions of volicitin, *N*-linolenoyl-L-glutamine, *N*-hydroxylinolenoyl-L-glutamic acid, *N*-linolenoyl-L-glutamic acid were *m/z* 421.27 (calculated for $C_{23}H_{37}N_2O_5^-$, 421.2702), 405.27 (calculated for $C_{23}H_{37}N_2O_4^-$, 405.2753), 422.27 (calculated for $C_{23}H_{36}NO_6^-$, 422.2543), 406.27 (calculated for $C_{23}H_{36}NO_5^-$, 406.2593), respectively. MS data were processed with LCMS solution ver. 3.4 software (Shimadzu).

Results and Discussion

From the thirteen non-lepidopteran insect species analyzed by LCMS, we determined that crickets (two species) and the fruit fly possess several kinds of FAAs (Table 1). These compounds were identified by comparison of their retention times and quasi-molecular ions with previously identified FAAs (standards) obtained from regurgitants of larval *S. litura* (Mori et al. 2003) and *M. sexta* (Alborn et al. 2003). *N*-Linolenoyl-L-glutamic acid (compound 7) was the main FAA component in extracts of *T. taiwanemma* (16.6±3.4 μg/insect, *N*=3) and *D. melanogaster* (8.9±1.7 μg/20 larvae, *N*=4). The quantity of *N*-linolenoyl-L-glutamic acid per mass of individual larva from each species was comparable with reported values for *M. sexta* (Alborn et al. 2003) or *N*-linolenoyl-L-glutamine in *S. litura* (unpublished).

For identification of FAAs found in crickets and fruit fly larvae, LCMS-IT-TOF analysis was conducted to obtain additional structural information. The following fragmentation ions were tentatively assigned: Volicitin, obtained from the regurgitant of larval *S. litura*, produces daughter ions at *m/z* 403.26 (dehydrated ion, calculated 403.2597), 385.25 (doubly dehydrated ion, calculated 385.2491), 315.24 (unknown), 231.21 (dehydrated and decarboxylated hydroxylinolenate ion, calculated 231.2113), and 145.06 (glutamine-derived ion, calculated 145.0614). *N*-Linolenoyl-L-glutamine produces daughter ions at *m/z* 387.27 (dehydrated ion, calculated 387.2648), 343.27 (further decarboxylated ion, calculated 343.2749), 233.23 (decarboxylated linolenate ion, calculate 233.2269), and 145.06 (glutamine-derived ion). *N*-Linolenoyl-L-glutamic acid, obtained from *M. sexta* regurgitant, produces daughter ions at *m/z* 388.25 (dehydrated ion, calculated 388.2488), 362.27 (decarboxylated ion, calculated 362.2695), and 277.22 (linolenic acid-derived ion, calculated 277.2168). The fragmentation pattern of FAAs containing glutamine, such as volicitin and *N*-linolenoyl-L-glutamine, typically includes a common glutamine-derived ion. Further MS analysis included manual scanning for daughter ions at *m/z* 277.22, 279.23, and 145.06, corresponding to other linolenoyl-, linoleoyl-, or glutamine conjugates. However, we could not detect any ions suggesting the presence of the related conjugates.

Figure 1 shows MS total ion chromatograms (TICs) with extracted ion chromatograms (XICs) of FAAs obtained from *T. taiwanemma* and *D. melanogaster* and MS^2 mass spectra with corresponding structures of FAAs obtained from *D. melanogaster* larvae. The spectra of *N*-linolenoyl-L-glutamic acid (d) and *N*-linolenoyl-L-glutamine (c) were nearly identical to those of authentic standards. Furthermore, *N*-linoleoyl-L-glutamic acid (e) was identified by its fragmentation pattern, which agreed reasonably with that of *N*-linolenoyl-L-glutamic acid (d). As the volicitin content was too low to yield fragment ions with enough intensity to measure accurately, only the dehydrated ion (*m/z* 403) and glutamine-derived ion (*m/z* 145) were detected along with ions from impurities (a). Spectrum (b) represents an

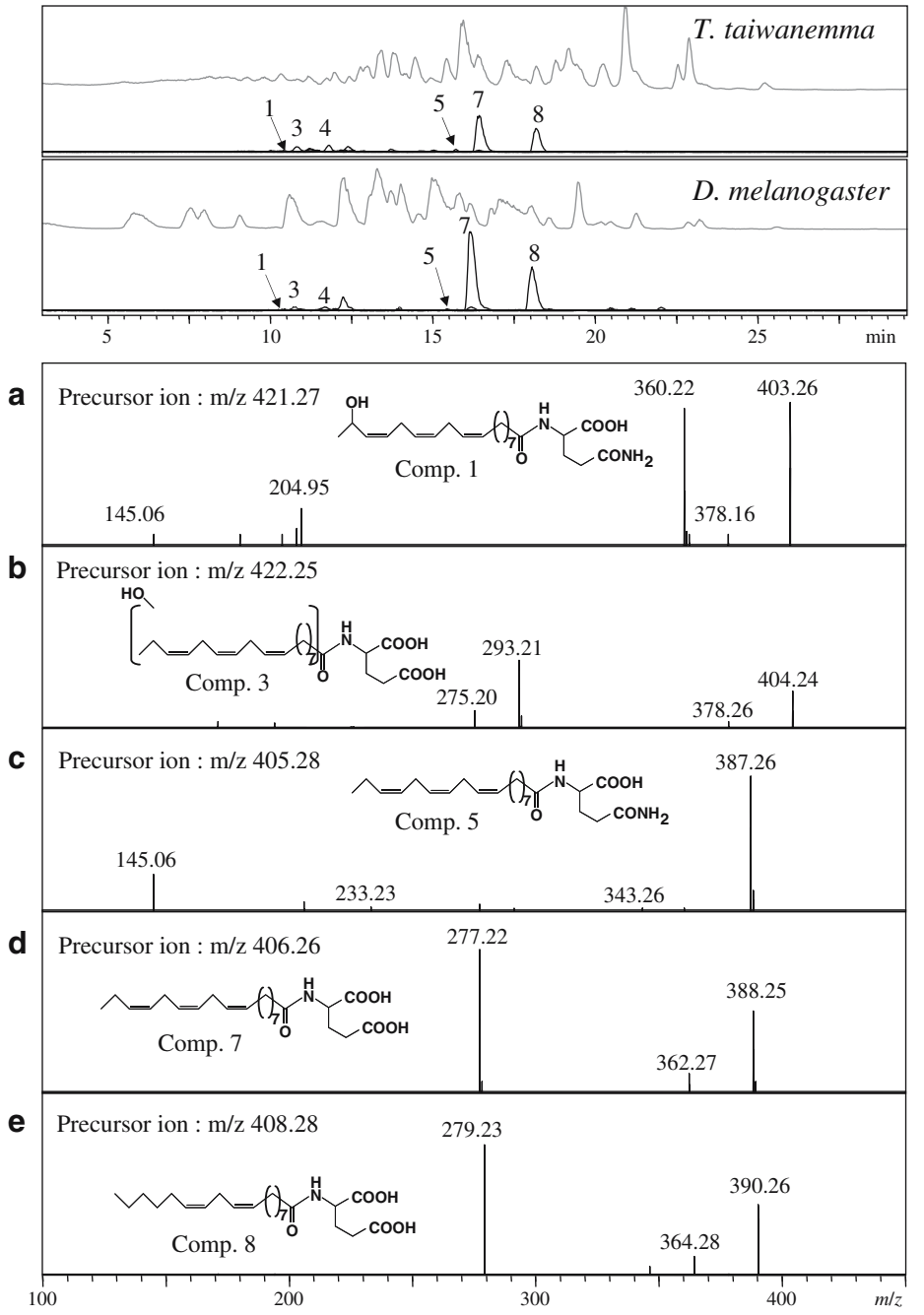


Fig. 1 Mass spectral total ion chromatograms and extracted ion chromatograms of FAAs obtained from *T. taiwanemma* and *D. melanogaster* and MS^2 spectra and structures of **a** volicitin, **b** *N*-hydroxylinolenoyl-L-glutamic acid, **c** *N*-linolenoyl-L-glutamine, **d** *N*-linolenoyl-L-glutamic acid, and **e** *N*-linoleoyl-L-glutamic acid found in the *D. melanogaster* larval extracts (structural analysis incomplete). The compound numbers represent the same compounds as those in Table 1

incompletely characterized FAA, which is thought to be *N*-hydroxylinolenoyl-L-glutamic acid (theoretical *m*/*z* 422.2543), as a presumed quasi-molecular ion appears at *m*/*z* 422.25. The fragmentation pattern agreed with the expected structure; dehydrated ion at *m*/*z* 404.24, doubly dehydrated ion at *m*/*z* 378.26, hydroxylinolenic acid-derived ion at *m*/*z* 293.21, and its dehydrated ion at *m*/*z* 275.20. When *D. melanogaster* larvae were reared on linolenic acid-supplemented artificial diet, peaks corresponding to FAAs containing a linolenic acid moiety had greater intensities and were more easily identified. However, it is not clear whether these FAAs came only from the larval gut. A compound with the same mass fragment patterns (not shown) was also found in the midgut contents of both cricket species. The absolute configuration and position of hydroxylation of the fatty acid moiety in the hydroxylated FAAs have yet to be confirmed. However, these findings are significant, as they indicate that FAAs might have physiological roles in additional orders of insects besides Lepidoptera. Halitschke et al. (2001) postulated that FAAs might simply act as biosurfactants or oil emulsifiers in the caterpillar gut, and indeed, this hypothesis may be applicable to crickets and fruit flies. However, the facts that FAAs identified thus far contain glutamic acid and/or glutamine, but not other amino acids, and hydroxylated FAAs are present in species from at least three different orders, suggest that a specific rather than general function in insect metabolism could also exist. As yet, we have no definitive explanation for their presence.

Acknowledgements This study was partly supported by a Grant-in-aid for Scientific Research (no. 15580090) and the 21st century COE program for Innovative Food and Environmental Studies Pioneered by Entomomimetic Sciences, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Naoko Yoshinaga is the recipient of a research fellowship (no. 01212) from the Japan Society for the Promotion of Science for Young Scientists.

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life for myrmecophiles is also dangerous, as ants are usually highly aggressive toward non-colony members. One way to avoid aggression by ants is to mimic the ants' chemical communication code, i.e., chemical mimicry. According to Vane-Wright (1976) and Dettner and Liepert (1994), chemical mimicry is characterized by an organism (mimic) that simulates volatile or nonvolatile chemical signals of a second living organism (model), which in turn are perceived as signals of interest by a third living organism (operator). In this way the mimic gains fitness because the operator identifies it as an example of the model. In the ant–myrmecophile system, the myrmecophile acts as the mimic, and the ant acts both as model and operator (bipolar system).

The cues generally used in chemical mimicry by myrmecophiles are cuticular hydrocarbons (CHC). In social insects, these substances are generally considered to be the main cues used for nest-mate recognition (Howard and Blomquist 2005). A number of studies indicate that myrmecophilous insects accumulate CHCs on their body surface that qualitatively and quantitatively resemble the CHC pattern of their host ants, thereby manipulating the nest-mate recognition system, and achieving integration into the host colony (e.g., Akino 2002; Elgar and Allan 2004; see reviews by Dettner and Liepert 1994; Howard and Blomquist 2005). Despite the advantages, chemical mimicry of CHCs is also problematic, as it restricts the myrmecophilous insect to just one host nest. Integration into other nests (e.g., to find mates) is risky because adaptation to a new nest-specific CHC pattern takes time, during which the intruder is detectable (Akino et al. 1996).

The imitation of CHCs used in nest-mate recognition might not be the only way for myrmecophilous insects to deceive their hosts. Kistner and Blum (1971) suggested that rove beetles of the genus *Pella* (Staphylinidae: Aleocharinae) produce and release alarm pheromone compounds of their host ants to their own advantage. This hypothesis was based on the observation that crushed individuals of two species of *Pella* and their host ant *Lasius spathebus* gave similar olfactory profiles (Kistner and Blum 1971). Later, this idea was supported by studies that revealed the presence of undecane and 6-methyl-5-hepten-2-one (sulcatone) in the tergal gland secretions of other myrmecophilous *Pella* species (Steidle and Dettner 1993). A tergal gland is present in most myrmecophilous and free-living beetles of the Aleocharinae and is used for defense against ants and other predators (Steidle and Dettner 1993). Undecane and sulcatone occur in two alarm pheromone glands of the beetle's host ant, *Lasius fuliginosus*, the Dufour and the mandibular gland, respectively (Maschwitz 1964; Bernardi et al. 1967). *Pella* beetles, formerly included in the genus *Zyras* (Kistner 1971; Maruyama 2006), live along the foraging trunk routes of *L. fuliginosus* where they prey on living ants or feed on dead ants present in the ants' garbage dumps. When encountering ants, these beetles either show a death-feigning position or, more frequently, present their abdominal tip to the ants. Generally, this behavior prompts the ant to inspect the abdominal tip and allows the beetle to escape. Only in persistent, aggressive inspections does the beetle release its tergal gland secretion, which repels the attacking ant (Hölldobler et al. 1981). This interaction raises the question, how does the beetle gain an advantage from the release of ant alarm pheromone compounds in this situation?

Generally, alarm pheromones in ants serve two different functions (Wilson and Regnier 1971). One of them, the "aggressive alarm", leads to recruitment of more workers and intensified attacks on intruders. The other, the "panic alarm", leads to escape, dispersion, and flight. Whereas the release of aggressive-alarm pheromones seems to be counterproductive for the beetles, the mimicry of panic-alarm pheromones could provide immediate short-term protection in aggressive interactions. This mode of chemical mimicry contrasts with the chemical mimicry of CHCs, which are nonvolatile and provide sustained acceptance in a host colony. This is analogous to volatile "propaganda" pheromones, which are involved in

interspecific interactions of ant species, like slave-making and nest usurpation (Regnier and Wilson 1971; Lenoir et al. 2001).

Experiments by Dumpert (1972) showed that undecane induces aggressive-alarm behavior in *L. fuliginosus.* We hypothesized that sulcatone could mimic a panic-alarm pheromone in *L. fuliginosus* when used by *Pella* beetles to repel their attacking hosts. To examine this hypothesis we: (1) re-analyzed the contents of the tergal gland secretions of two *Pella* beetles, *P. funestus* and *P. humeralis*, and studied the effect of (2) pure undecane and sulcatone, and (3) sulcatone in combination with other compounds of the tergal gland secretions on workers of *L. fuliginosus* in laboratory and field experiments.

Methods and Materials

Insects Beetles were obtained from eight different colonies of *L. fuliginosus* in southwest Germany by collecting substrates around ant nests and sorting out litter on a white underground. The beetles were reared in plastic Petri dishes (diameter 90 mm) at room temperature under daylight (Schminke 1978). Petri dishes were filled with a 5-mm plaster layer, which was moistened daily to maintain humidity. In addition, a small piece of filter paper was provided as shelter. Beetles were fed every second day with dead *L. fuliginosus* workers. *L. fuliginosus* ants were collected in the field at the same sites as the beetles, reared in plastic containers (50.2×35.5×17 cm or 50.2×35.5×25.5 cm), and supplied with aqueous sucrose or honey solution (Maschwitz and Hölldobler 1970). Bhatkar diet was provided every second day to supply the ants with proteins, minerals, and vitamins (Bhatkar and Whitcomb 1970). All insects were identified to species level with the identification keys by Freude et al. (1974) for beetles and Seifert (1996) for ants.

Chemical Analysis of the Tergal Gland Secretions and Ant Alarm Pheromones Volatile tergal gland secretions of the beetles and ant pheromones were collected from the headspace of the organisms by using a solid-phase microextraction (SPME) needle coated with 65 μm Polydimethylsiloxane/Divinylbenzene (Arthur and Pawliszyn 1990). Single beetles were placed in a 60-ml glass flask, and the SPME-fiber was inserted through a rubber plug. To stimulate the release of tergal gland secretion, beetles were stimulated with a magnetic stir bar, which was carefully moved from the outside of the flask toward the beetles with a magnetic stick. After a 10-min adsorption time, the fiber was removed from the flask and placed for 2 min into the heated (250°C) injection port of an Agilent Technologies 6890 gas chromatograph (GC) coupled with a 5973 network mass selective detector. The GC was equipped with a fused-silica-capillary column (HP 5, 30 m×0.2 mm, 0.5 μm film thickness) and programmed as follows: 60°C for 3 min., 60°C to 300°C at 3°C min^{-1}. Helium, at a constant 1.6 ml min^{-1}, was the carrier gas. Chromatographs and mass spectra were analyzed by Enhanced Chemstation software (Agilent technologies) by using Wiley 275 and NIST 2002 databases. For quantitative analyses, calibration curves with synthetic undecane (Aldrich Chemical Co.; purity 99+%), *p*-benzoquinone (Merck & Co;, purity: ≥98%), *p*-toluquinone (Merck & Co., purity: >98%), and 6-methyl-5-hepten-2-one (Aldrich Chemical Co., purity: 99%), from 1 to 30 μg, were generated. The calibration curve of *p*-toluquinone was used to estimate the amount of 2-methoxy-3-methyl-1,4-benzoquinone. Data obtained from these analyses were used to estimate the appropriate concentrations to be used in the bioassays. Six specimens were analyzed for each beetle species. To validate the artificial stimulation, the analysis was repeated with

single beetle specimens together with three workers of *L. fuliginosus*. To study the release of alarm pheromones by the ants, three workers of *L. fuliginosus* were tested under the same conditions.

Role of Sulcatone and Undecane as Alarm Pheromones In a first experiment, we determined whether sulcatone acts as an attractant or repellent on *L. fuliginosus* workers by using feeding tests in the laboratory. Thirty workers of *L. fuliginosus* were placed in a Petri dish without food. After 2 d, an aqueous solution of sucrose (1:1 *w*/*w*) was placed on a feeding platform in the center of the Petri dish next to a piece of filter paper (Whatman Grade I). When at least 10 ants were feeding simultaneously on the sugar water, 10 μl of sulcatone (0.1 μg/μl dissolved in hexane) were applied to the filter paper, and the number of ants at the feeding source counted every 15 sec for 120 sec.

To test the potential alarm pheromone effect of sulcatone and undecane on ant workers in the field, filter paper balls (1 × 1 cm; Whatman Grade I) were treated with 10-μl test solutions and placed on natural foraging trunk routes of *L. fuliginosus*, some 0.5–1.5 m from the nest entrance. Test solutions consisted of undecane (0.42 μg/μl in hexane), sulcatone (0.13 μg/μl in hexane), or hexane (control). The reactions of ants were videotaped for 90 sec, and analyzed later. Behavior was considered aggressive when ants touched the filter paper ball with both antennae and open mandibles or when they were biting into it. Antennation, i.e., touching the filter paper ball with both antennae and closed mandibles, was considered as nonaggressive behavior. The number of ants on the trunk trails varied during the different experimental dates at the different sites. Therefore, the number of passing ants was recorded for 1 min before each test and used to calculate the percentage of reacting ants in relation to the overall number of passing ants.

Effect of Sulcatone Added to Other Compounds Found in the Tergal Gland In the first experiment in the laboratory, 10 ants were placed in a Petri dish with a filter paper ball (see above) in the center. The filter paper ball was treated with one of the following test solutions in hexane: (1) undecane (0.42 μg/μl); (2) *p*-toluquinone (0.39 μg/μl) plus *p*-benzoquinone (0.06 μg/μl); (3) undecane (0.42 μg/μl) and sulcatone (0.13 μg/μl); (4) *p*-toluquinone (0.39 μg/μl), *p*-benzoquinone (0.06 μg/μl), and sulcatone (0.13 μg/μl); (5) hexane (control). A second experiment was performed in the field with filter paper balls placed on foraging trunk routes of *L. fuliginosus* as described above. Two different mixtures (Mix+ and Mix−) of the main tergal gland secretion compounds were applied to the filter paper balls. The concentrations used matched the average composition of the tergal secretion of the two *Pella* species found during beetle–ant interactions in the laboratory. Mix+ consisted of *p*-benzoquinone (0.06 μg/μl), *p*-toluquinone (5.05 μg/μl), undecane (3.09 μg/μl), sulcatone (1.46 μg/μl), and tridecane (0.15 μg/μl). Mix− was similar to Mix+ but without sulcatone. Hexane was used as a control.

Statistics The results of the behavioral assays were analyzed by Mann–Whitney *U* tests followed by sequential Bonferroni correction using the software package STATISTICA 99-Edition. (Stat Soft Inc.)

Results

Chemical Analysis of Tergal Gland Secretions and Ant Alarm Pheromones Twelve different substances were identified in the headspace collections of *Pella* beetles after stimulation with

either a magnetic stir bar or by aggressive ant–beetle interactions. In both *Pella* species, large amounts of sulcatone, *p*-toluquinone, undecane, and 2-methoxy-3-methyl-1,4-benzoquinone were found (Fig. 1, Table 1). Large amounts of undecane and dendrolasin were found in the headspace collections of *L. fuliginosus* workers that had been teased with a magnetic stir bar. Additionally, tridecane, citronellal, neral (*cis*-citral), and geranial (*trans*-citral) were detected, whereas sulcatone was found in low amounts (Fig. 2).

Role of Sulcatone and Undecane as Alarm Pheromones In the feeding experiment, when chemicals were applied to the filter paper, most ants left the platform after application. When

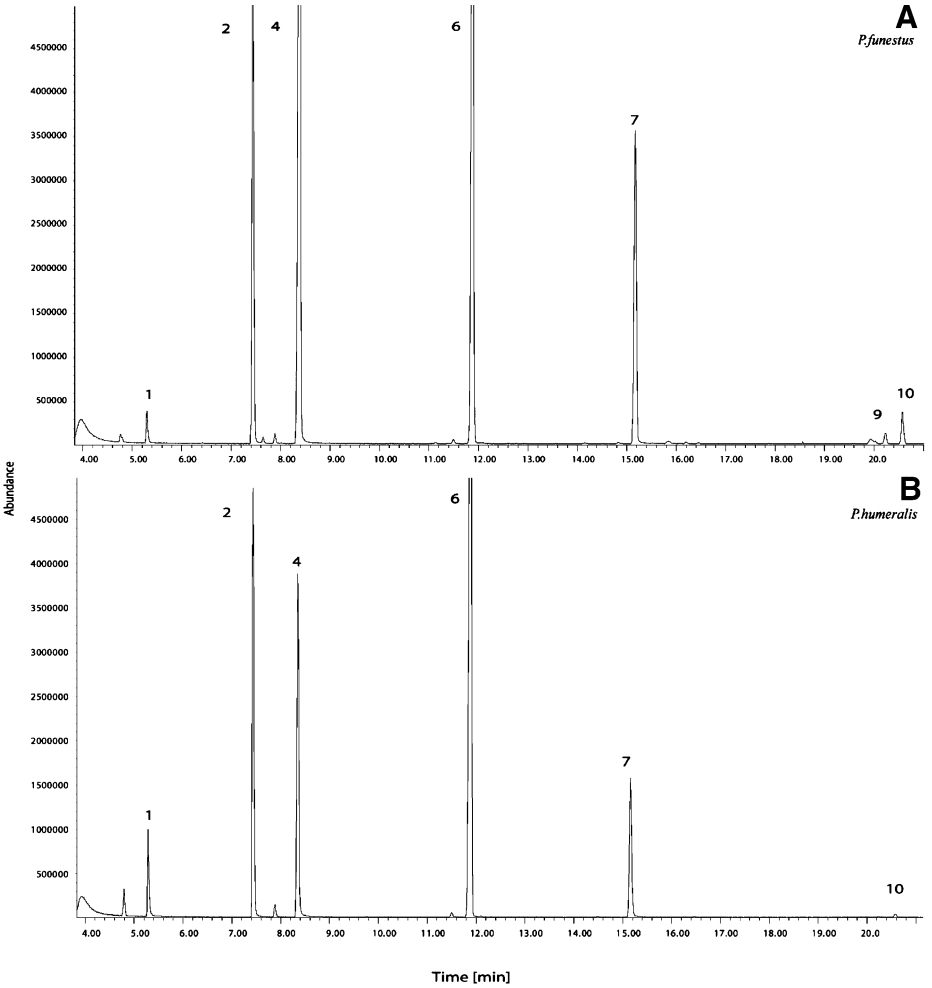


Fig. 1 Total ion chromatograms of the tergal gland secretions obtained by stir bar irritation of two *Pella*species (A: *P. funestus*; B: *P. humeralis*). The numbers in the chromatograms refer to the numbers of the chemicals in Table 1

Table 1 Chemicals found in the headspace of a flask containing rove beetles of the genus *Pella*, which have been stimulated by either a moving magnetic bar (A), or aggressive interactions with workers of *Lasius fuliginosus* (B); (C) substances found by analysis of dissected glands in a previous study (Steidle and Dettner 1993)

Substances		*P. funestus*			*P. humeralis*		
		A	B	C	A	B	C
		N=6	*N*=3		*N*=6	*N*=2	
1	*p*-benzoquinone	0.6±0.2[a]	0.1±0.2	–[b]	5.1±1.7	1.2±0.7	–
2	Sulcatone (6-methyl-5-hepten-2-one)	14.6±3.9	13.3±9.0	+	18.1±6.6	7.0±5.1	+
3	*n*-decane	0.3±0.1	0.2±0.2	–	0.5±0.2	–	–
4	*p*-toluquinone	34.1±6.8	12.4±13.2	–	21.5±5.4	9.5±4.5	+
5	Undecene	0.2±0.9	–	–	0.2±0.1	–	–
6	Undecane	30.9±10.1	52.5±24.1	+	44.2±10.8	49.9±13.1	+
7	2-methoxy-3-methyl-1,4-benzoquinone	16.5±4.6	3.3±4.1	+	9.5±1.2	30.7±24.9	+
8	Hydroquinone	–	0.3±0.2	–	–	–	+
9	Tridecene	0.6±0.6	0.2±0.2	–	0.2±0.1	0.5±0.5	–
10	Tridecane	1.5±1.1	2.3±1.9	+	0.2±0.1	1.3±1.0	+
11	*p*-toluhydro-quinone	–	15.1±18.9	+	–	–	+
12	Dodecanal	0.2±0.1	–	+	0.3±0	–	–
13	2-methoxy-3-methyl-1,4-hydroquinone	–	–	+	–	–	+
14	Pentadecane	–	–	+	–	–	+
15	*n*-heptadecene	–	–	–	–	–	+
16	Isovaleric acid	–	–	–	–	–	+

[a] Peak area (mean ± SD)

[b] Substances found (+) or not found (−) previously in a study by Steidle and Dettner (1993)

hexane was applied to the filter paper, all the ants returned within 30 sec and resumed feeding for about another 30 sec, after which the number of ants decreased. When sulcatone was applied, only about half the initial number of ants returned to the feeding platform and the number remained low for the duration of the experiment (significantly, $P<0.05$, lower than for the controls for up to 90 sec after the start), suggesting that sulcatone had a repellent effect on workers of *L. fuliginosus* (Fig. 3). In the field experiment, the percentage of ants that antennated paper balls impregnated with sulcatone was significantly lower than the number of ants that antennated filter paper balls with hexane or undecane (Fig. 4A). In addition, undecane stimulated significantly more aggressive behavior than hexane, whereas sulcatone stimulated significantly lower amounts of aggressive behavior than hexane (Fig. 4B). This is consistent with sulcatone having a repellent effect on workers of *L. fuliginosus*, but suggests that undecane stimulates increased aggression.

Effect of Sulcatone Added to Other Compounds Found in the Tergal Gland When quinones or undecane were mixed with sulcatone, the observed aggressive behavior of ants was reduced to a level comparable to that of the control (Fig. 5). In the field experiment, both mixtures (Mix+, Mix−) of the main tergal gland compounds elicited stronger aggressions toward the filter paper balls than the control (Fig. 6). However, less aggressive behavior was observed to the mixture with sulcatone (Mix+) than to the mixture lacking this chemical (Mix−).

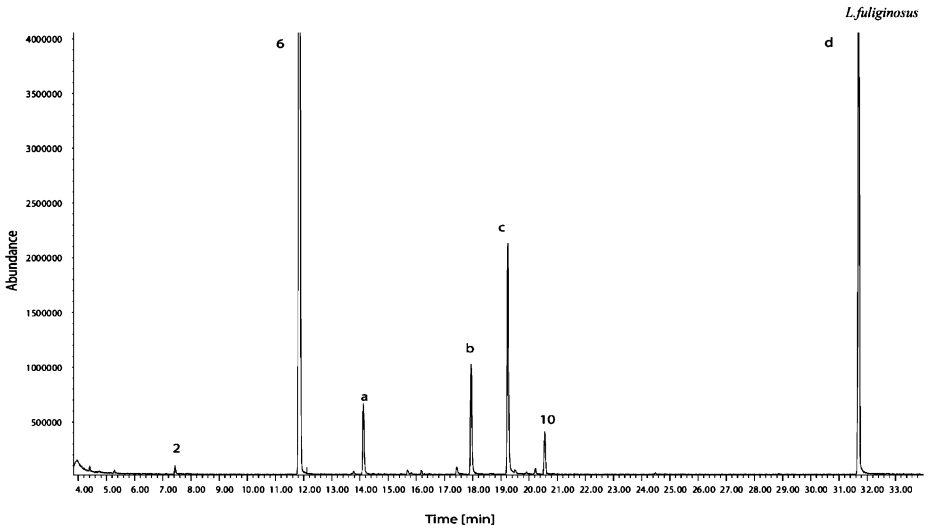


Fig. 2 Total ion chromatogram of volatile chemicals released by *L. fuliginosus* workers during stir bar stimulation. The numbers in the chromatogram refer to the numbers of the substances in Table 1. Substances found only in *L. fuliginosus* are indicated by letters a–d (a: citronellal; b: neral; c: geranial; d: dendrolasin)

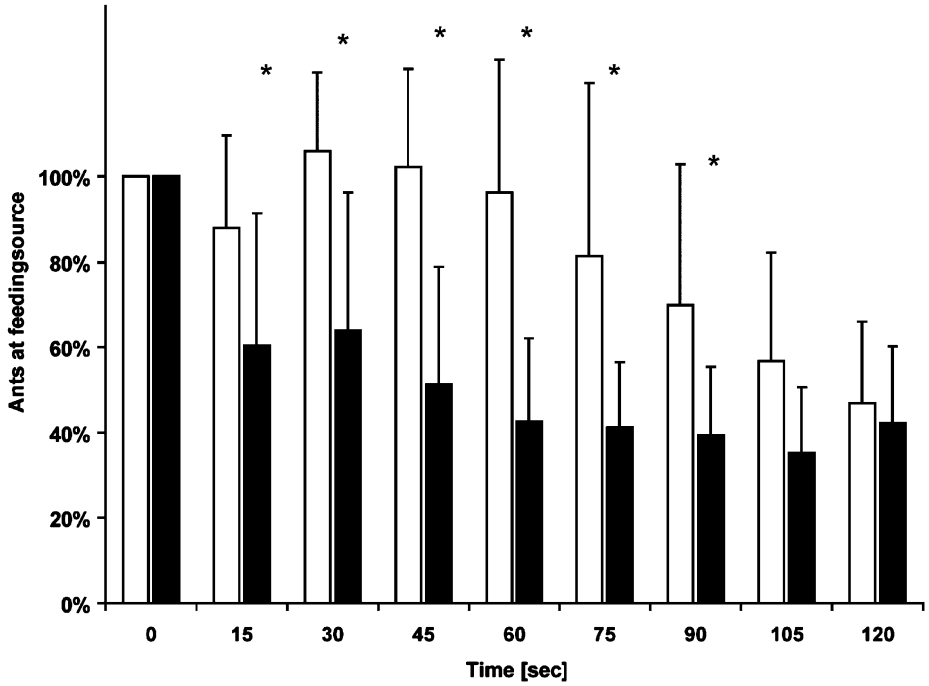


Fig. 3 Number of feeding ants at a sugar–water source during a laboratory experiment. White bars: control experiment (mean ± SD) in which hexane was applied next to the sugar water; black bars: test experiment (mean ± SD) in which sulcatone dissolved in hexane was applied next to the sugar water (*$P \leq 0.05$; $N=10$; Mann–Whitney U test)

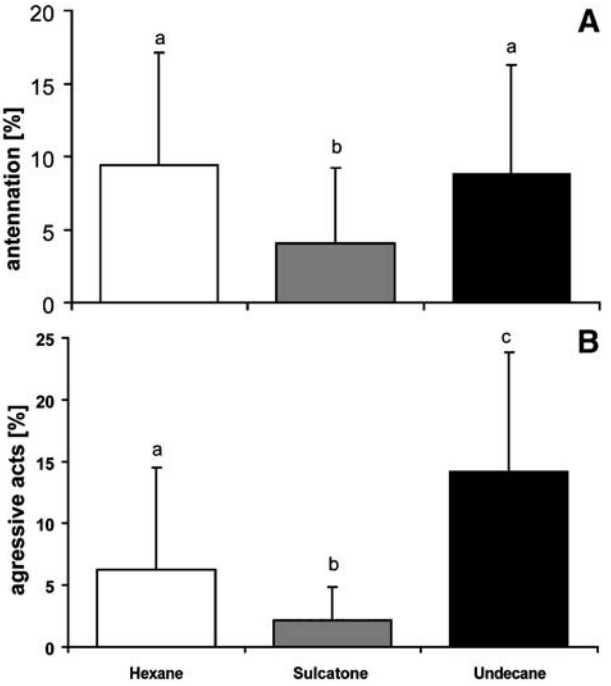


Fig. 4 A and B: Percentage of total passing ants (mean ± SD) on a foraging trunk route exhibiting behavior (A: antennation; B: aggression) to a filter-paper ball treated with hexane (control), sulcatone, or undecane. Bars with different letters are significantly different ($P \leq 0.001$; $N=50$; Mann–Whitney U test)

Discussion

Twelve compounds, released by *P. funestus* and *P. humeralis* beetles upon being stimulated with a magnetic stir bar or after interactions with worker ants of the species *L. fuliginosus*, were identified. The chemical profiles obtained from beetles stimulated by the stir bar or by interaction with ants were similar, indicating that treatment with the stir bar is an appropriate method to study the tergal gland chemicals released in defensive situations by *Pella* beetles.

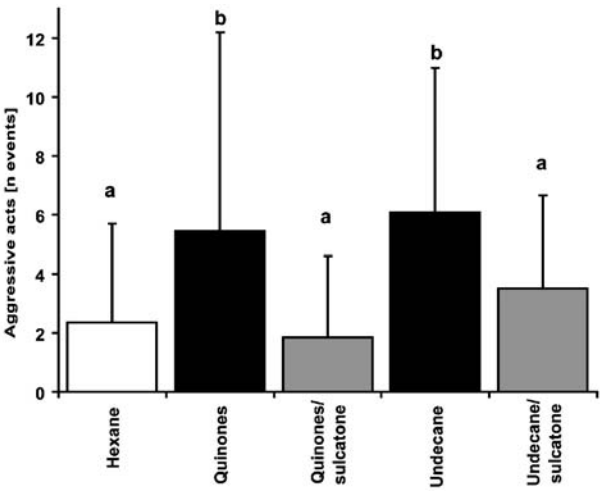


Fig. 5 Number of aggressive acts by ants (mean ± SD) in a laboratory experiment toward a filter-paper ball treated with different chemicals found in the tergal gland secretions of *Pella* rove beetles. Bars with different letters are significantly different ($P \leq 0.05$; $N=30$; Mann–Whitney U test)

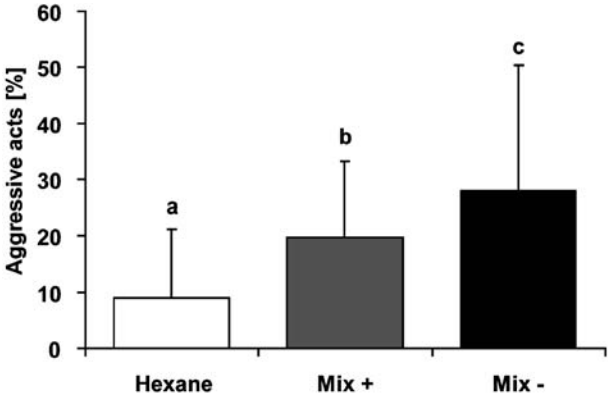


Fig. 6 Percentage of passing ants (mean ± SD) on a foraging trunk route exhibiting aggressive acts toward a filter-paper ball treated with a mixture of quinones, undecane, and sulcatone (Mix+) compared to a similar mixture without sulcatone (Mix−). Bars with different letters are significantly different ($P \leq 0.05$; $N=56$; Mann–Whitney U test)

There are some discrepancies between the present study and the study of Steidle and Dettner (1993), in which the tergal gland chemistries of 20 species of Aleocharinae, including *P. funestus* and *P. humeralis*, were studied. In contrast to the SPME-headspace method employed in the present study, Steidle and Dettner (1993) placed excised glands in the injection port of the GC. We presume that this methodological difference was responsible for our nondetection of some compounds of low volatility (pentadecane, *n*-heptadecene, 2-methoxy-3-methyl-1.4-hydroquinone) found by Steidle and Dettner (1993). The present study also found several volatile compounds (*n*-decane, undecene, tridecene), not reported by Steidle and Dettner (1993). These substances occur only in smaller amounts, and may have evaporated during the preparation of the tergal glands in the earlier study. Given that, volatile chemicals, rather than compounds of low volatility, probably contribute more to the effect of tergal gland secretions in aggressive interactions, the results from the SPME headspace method are probably more indicative of the defensive chemicals released by the insects than are the data from the excised-gland method.

Regardless of the method used, the main compounds in the defensive secretions of both *Pella* species studied are sulcatone, *p*-toluquinone, undecane, and 2-methoxy-3-methyl-1,4-benzoquinone. Of these, the quinones and undecane can be found in defensive tergal gland secretions of many Aleocharinae (Steidle and Dettner 1993). As demonstrated by topical application experiments with fly larvae, quinones are the main toxins in the secretion, whereas undecane serves as a solvent for the quinones (Steidle and Dettner 1993). Sulcatone has been found only in the myrmecophilous genera *Pella* and *Lomechusa* of the Aleocharinae and not in free-living species to date.

Chemical analyses of the headspace from disturbed workers of *L. fuliginosus* showed the release of undecane, dendrolasin, tridecane, citronellal, neral, geranial, and sulcatone. Undecane has previously been reported as an alarm pheromone in *L. fuliginosus* (Dumpert 1972), and this function was supported by our laboratory and field experiments with *L. fuliginosus* workers. Sulcatone has been previously identified as a component of the mandibular gland secretion (Bernardi et al. 1967), which is considered to contain alarm pheromones (Wilson and Regnier 1971), although no function of sulcatone in *L. fuliginosus* has been reported. Sulcatone has also been described as an alarm pheromone in *Formica* ant species of the same subfamily as *L. fuliginosus* (Duffield et al. 1977) and also in *Iridomyrmex* and *Tapinoma* (Hölldobler and Wilson 1990). In our laboratory feeding experiments, sulcatone repelled a significant number of *L. fuliginosus* workers from an otherwise attractive feeding

source. In the field experiments, it had a general repellent effect. Therefore, we conclude that in *L. fuliginosus*, sulcatone serves as a panic alarm pheromone *sensu* Wilson and Regnier (1971), inducing avoidance and flight and overruling the effect of the aggression-inducing pheromone undecane. It might be released in encounters with superior aggressors or in situations where there are no benefits in fighting, e.g., at remote feeding sites.

Undecane and sulcatone occur in the tergal gland secretions of *P. funestus* and *P. humeralis* and are also released by excited workers of the host ant *L. fuliginosus*. What, then, is the function of sulcatone released by *Pella* rove beetles? Our laboratory and field experiments showed that not only undecane but also a mixture of quinones induce aggression in *L. fuliginosus* workers. However, when sulcatone was added to quinones or undecane in the laboratory and field experiments, there was a reduction in aggression by *L. fuliginosus* workers. Therefore, release of sulcatone blocks the effect of the aggression-inducing chemicals and enables the beetles to use their tergal defensive gland secretion to repel an aggressive ant without inducing more aggression.

In summary, our results support the long-standing hypothesis by Kistner and Blum (1971), formulated for two Japanese *Pella* species, that myrmecophilic insects mimic alarm pheromones of their hosts. Furthermore, the reported case of these *Pella* beetles represents a rare case of chemical mimicry (according to the definition in Dettner and Liepert 1994) in myrmecophilous insects that does not involve cuticular hydrocarbons but other pheromonal cues. It should be noted that chemical mimicry of the alarm pheromone is not the only strategy used by beetles in the genus *Pella* to avoid aggressive interactions with host ants. Other strategies involve behavioral adaptations (e.g., death-feigning), the use of the appeasement gland (Hölldobler et al. 1981), and chemical mimicry of CHC patterns (Akino 2002). From an evolutionary perspective, it is unclear whether the production of sulcatone is a plesiomorphic pre-adaptation enabling the switch to myrmecophily in *Pella*, or whether it has been developed as an apomorphic trait when the beetles were already integrated in ant nests. Interestingly, not all myrmecophilic species of the genus *Pella* produce sulcatone (Stoeffler, unpublished), but it is also found in the highly myrmecophilic species *Lomechusa strumosa*, which belongs to the same tribe Myrmedoniini (Steidle and Dettner 1993). More work including molecular studies to define relationships within the Myrmedoniini is required to determine how myrmecophily and the production of sulcatone evolved in rove beetles of the genus *Pella*.

Acknowledgments We thank Cornelia Gantert for continuous support in the establishment and maintenance of the ant colonies used in this study. Andrea Fuhrmann, Angela Hücklhoven, and Stine Mencl contributed by performing behavioral experiments. The manuscript was improved, thanks to comments of anonymous reviewers and the revision by Dr. Astrid Groot and Simone Kienle.

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probable genetic component associated with choice, as insects feeding on corn (*Zea mays* L.) are genetically different from those feeding on mugwort (*Artemisia vulgaris* L.) and hop (*Humulus lupulus* L.; Bethenod et al. 2005; Malausa et al. 2005). Females use plant odors to avoid damaged maize plants (Schurr and Holdaway 1970) or to select some maize hybrids over others (Lupoli et al. 1990), as well as to discriminate among plant species (Udayagiri and Mason 1995). Certain odors are even repellent to them (Binder et al. 1995; Binder and Robbins 1997). Some studies have also documented how contact chemicals, like sugars (Derridj et al. 1986) and surface waxes (Udayagiri and Mason 1997), affect host plant choices and oviposition. These observations suggest that the chemosensory system of ECB females is sensitive to a number of host and nonhost plant chemicals and that despite its large range of hosts, they monitor volatile and contact chemicals that allow them to select the proper plant and avoid "unsuitable" hosts.

In selecting or avoiding a potential host plant, do females use the same stimuli as their larvae? Although some studies have reported that adult females may use stimuli different from larvae to select their host (Roessingh et al. 2000), others suggest that larvae and adults use the same contact stimuli when accepting (Renwick 2002; Miles et al. 2005) or rejecting host plants (Qiu et al. 1998). We have recently shown that larval stages and adult females of the grapevine moth, *Lobesia botrana*, avoid 20-hydroxyecdysone (20E; Calas et al. 2006). 20E, the major molting hormone of insects, is the most common of the PEs, a structurally homogenous class of secondary substances produced by plants (Marion-Poll et al. 2005). PEs are considered to be plant defense compounds (Schmelz et al. 1999, 2000) that mimic the genuine insect hormone. They have been found in the seeds of 5–6% of 5,000 plant species sampled across different plant taxa (Dinan et al. 2001), ranging from primitive ferns, to the more evolved Angiosperms. Therefore, we propose that polyphagous insects like ECB will have been repeatedly exposed to PEs and consequently have evolved strategies to avoid plants using this family of defense compounds.

We have already shown that 20E is deterrent and toxic for ECB larvae (Darazy-Choubaya 2002) and that they have taste receptors that can detect 20E at a threshold of 10^{-6}–10^{-7} M (Marion-Poll and Descoins 2002). Therefore, we tested the hypothesis that ECB adult females would avoid laying eggs on 20E-containing substrates. We first determined if ECB females can detect 20E through neurons in taste sensilla located on the legs or on the ovipositor (Marion-Poll et al. 1992). We also monitored the number of eggs laid on different artificial substrates (filter paper, glass, and nylon) and plants treated with 20E, as well as timing the different sequences of behavior leading to egg laying when females were provided on treated or untreated glass oviposition sites. Our results indicate that ECB adult females detect 20E with their taste receptors and change the duration of certain oviposition behaviors. However, the number of eggs laid only declined when natural rather than artificial substrates were used. Several hypotheses are proposed to explain the differences observed.

Methods and Materials

Chemicals 20E, purified from *Leuzea carthamoides* (Willd.) DC. (Asteraceae; 95% minimum purity with minor ecdysteroids mixtures of polypodine B, ajugasterone C, and inokosterone), was obtained from SciTech (Prague, Czech Republic) and from Lafont (University of Paris VI). It was dissolved in ultrapure water or in ethanol depending on the bioassays. For electrophysiology, 20E was dissolved in 10^{-4} M potassium chloride (KCl, Prolabo) and used at concentrations ranging from 10^{-8} to 10^{-2} M.

Plants Individual maize *Centena* (Pioneer, Toulouse) plants were grown in plastic pots in a greenhouse at INRA Versailles and were used in assays when they had developed eight leaves.

Insects ECB pupae were obtained from INRA Le Magneraud. This strain is reared on an artificial medium and regularly renewed with wild insects. Pupae were maintained in plastic boxes at 25°C under a 16-hr light/8-hr dark photoperiod and 70% relative humidity until adults emerged. We used 24-hr-old virgin females for the electrophysiological studies, to ensure that good recordings were obtained.

Oviposition-choice tests and the observation of oviposition-related behaviors were performed in an environmental chamber (25°C; 70–80% relative humidity) under a 16-hr light/8-hr dark photoperiod. Couples of mature females and males (48 hr old) were placed in cages to mate, and females started to lay eggs at the beginning of the next night. We used this time as a pretest period to check if adults were mated, and the actual behavioral tests were performed at the beginning of the following scotophase.

Electrophysiology Before dissection, females were briefly anesthetized with carbon dioxide. For the recording from tarsal sensilla, females were decapitated by using fine scissors and then fixed on a polystyrene block with fine strips of tape. A glass capillary electrode, filled with Ringer solution, was inserted into the abdomen and connected to the ground. One of the prothoracic legs was taped to the support to expose the ventral side of the fifth tarsomere. Two types of taste sensilla were found on the tarsi of ECB adults, CRa and CRb (Marion-Poll et al. 1992), but as CRa sensilla did not respond to 20E (data not shown), measurements were only recorded from the CRb. For the recording from the ovipositor, the distal part of the abdomen was excised with a scalpel and placed on a small silicone dish filled with Ringer solution. Previous observations showed that there are about 12 taste sensilla of type CRa (30–40 μm) on the outer rim of each papilla, within a field of about 200 bristles (80–160 μm; Marion-Poll et al. 1992). These taste sensilla are identifiable under the microscope by their dark, blunt tip. Recordings were made from randomly selected sensilla.

For stimulation, we used glass capillary electrodes (borosilicate glass, outer diameter = 1.0 mm, inner diameter = 0.78 mm) pulled to a 10-μm-diameter tip (Narishige PC-10) filled just before the recording. The electrode was placed on the tip of a sensillum chaeticum by using a Leica MZ12 (×350). The electrical signal was preamplified (TastePROBE DTP-02, Syntech, The Netherlands), amplified (×1,000), and filtered (8-poles Bessel filter: 0.1–30 to 2,800 Hz; CyberAmp, 320, Axon Instruments). The electrical artifact generated at the moment of the contact was detected by the TastePROBE amplifier, which triggered a 2-sec data acquisition. Data were recorded and stored on a computer with a 16-bit A/D conversion card (DT9803 USB A/D; Data Translation, USA) under the control of a custom software, dbWave. Recordings were then analyzed with dbWave, to detect and sort spikes according to their amplitude and shape using interactive procedures.

Responses to the different stimuli were evaluated by counting the total number of spikes of the recording. Series of recordings were done starting with 10^{-4} M KCl (also used as electrolyte of nonconductive solutions) and followed by ascending concentrations of 20E (tarsal sensilla: decadic steps from 10^{-8} to 10^{-2} M; ovipositor: decadic steps from 10^{-6} to 10^{-3} M). Consecutive stimuli on a sensillum were applied at an interval of greater than 2 min to avoid adaptation. Data from the tarsi were collected from 16 insects ($N = 9$–37 recordings × 8 doses) while from ovipositors were collected from ten insects ($N = 12$–26 recordings × 5 doses).

Oviposition Tests on Artificial Substrates The oviposition cages were metal cylinders (height = 20 cm, diameter = 19 cm, 2.5-mm mesh). The top of each cage was closed with a paper, nylon, or glass (19 cm diameter) disk supported by a larger cloth (6-mm mesh). This cloth allowed females to hang on and touch the disk. The two opposing quarters of the disk received 200 μl of a solvent or 10^{-4} to 10^{-2} M of 20E. Ultrapure water was used as the solvent for nylon disks, while ethanol was used for glass and filter paper substrates. After letting the solvent evaporate for 20 min, a disk was placed in a cage containing five females and eight males. We performed ten replicates, each lasting an hour, for each substrate and each concentration.

Oviposition-related Behaviors on Glass Substrates At the beginning of the scotophase, mated females were introduced into small individual nylon cages (height = 12 cm; diameter = 10 cm), topped with a metal cloth (mesh size = 6 mm) and covered by a glass Petri dish (diameter = 11 cm). The surface of the Petri dish facing the inside of the cage was treated with 240 μl of either ethanol or 10^{-4} M 20E in ethanol. We recorded the oviposition behavior from above the cage, under red light, with a digital video camera recorder (Sony DCR-HC20). The behavioral sequences recorded (flying, walking, sweeping, and egg laying) correspond to those previously described for the ECB (Binder and Robbins 1996; Garnier-Geoffroy et al. 1996). Each experimental session lasted 2–3 hr. We monitored the behavior of ten females for each condition (control and 10^{-4} M 20E).

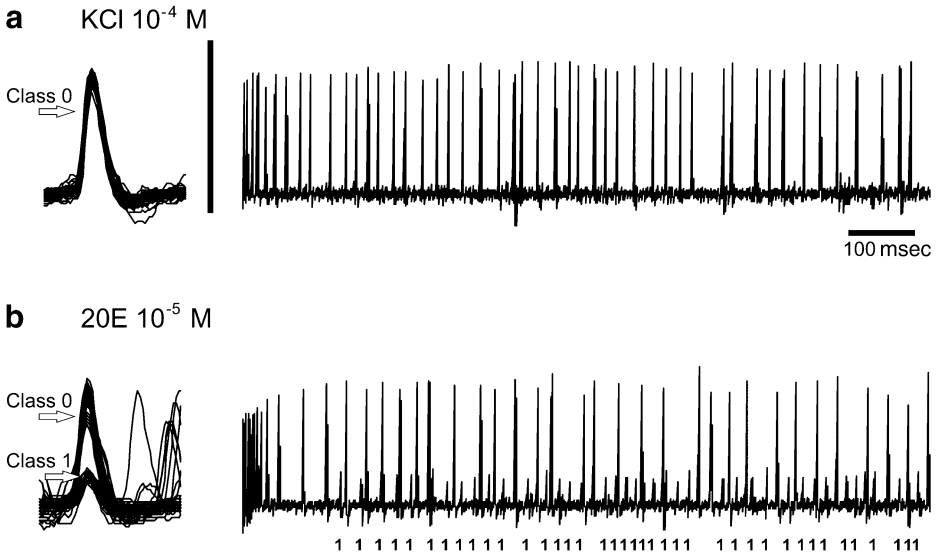
Oviposition Tests on Plants Maize plants were sprayed (Ecospray, VWR, France) with 15 ml of 10^{-4} M 20E or pure water. Half an hour later, when plants were dry, two plants (20E+control or control+control) were placed together in a plastic pot (diameter = 30 cm) filled with vermiculite. The plants were covered with a nylon cage (a conic section: top = 25 cm diameter, bottom = 40 cm diameter, height = 70 cm), and five females were introduced. Experiments were run for 2 hr at the beginning of the scotophase. The number of eggs deposited on each leaf of both plants was noted. The leaves were numbered 1 to 8, from the bottom to the top. We performed ten replicates for choice tests using 20E and five replicates for control tests (control vs. control).

Statistics Statistical tests were done with Minitab 12 software (Minitab, France). Electrophysiological responses were compared with the response to the electrolyte (0.1 mM KCl) using Mann–Whitney (MW)'s *U* test. Oviposition scores were compared by using nonparametric statistics with the Wilcoxon matched-pair signed rank test for choice experiments and the MW test for nonchoice ones.

Results

Taste Sensilla on the Tarsi In CRb sensilla, we found at least two classes of spikes in response to KCl and to 20E, which we called class 0 and class 1. Class 0 is characterized by medium amplitude spikes (1.4–1.8 mV, Fig. 1). Its firing activity was quite high, except at the highest concentration of 20E where it decreased by almost half (average rate: 40 vs. 23 spikes/s; P=0.005; Fig. 2). Although this class of spikes could originate from a "water" cell, its activity did not decrease in the presence of higher concentrations of salt (data not shown).

Class 1 has smaller amplitude spikes (0.5–0.8 mV) when activated by 20E (Figs. 1 and 2). When near the detection threshold, between 10^{-7} and 10^{-6} M, there was a response delay of 200–300 msec, which decreased at higher concentrations. The activity of this class of spikes


a
KCl 10-4 M
Class 0
100 msec
b
20E 10-5 M
Class 0
Class 1
1 1 1

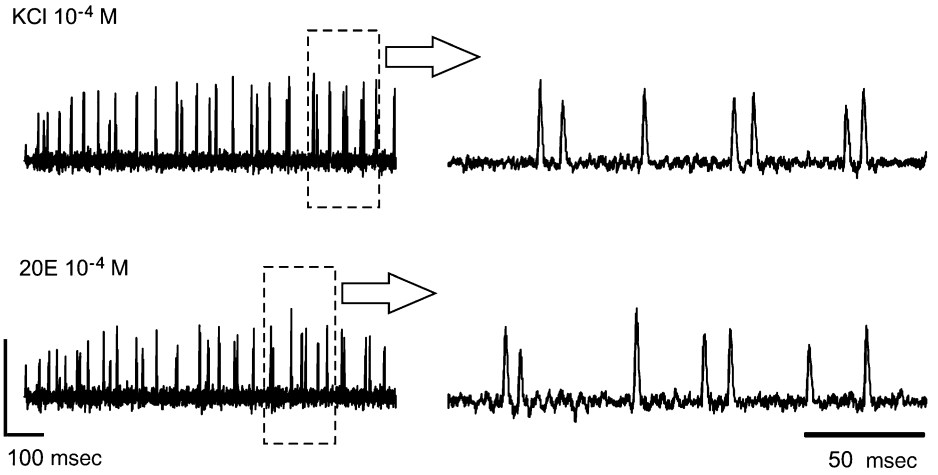


Fig. 3 Electrophysiological recordings from sensilla chaetica type CRa of the ovipositor of *Ostrinia nubilalis*. *On the left*, a 1-sec recording sample to KCl 10^{-4} M and 20E 10^{-4} M. *On the right*, an enlarged view of 200 msec, showing that several cells are active. The recordings look very similar at all concentration of 20E. Vertical bar=1 mV

Oviposition Tests on Artificial Substrates Females readily oviposit on all artificial substrates tested. In control vs. control assays, the total number of eggs (mean±s.e.m.) laid during the first hour was similar for glass (207.9±117.5, N=10), paper (201.6±116.4, N=14), and nylon (211.4±112.1, N=10). Similarly, in choice tests, there were no significant differences in the number of eggs laid on the treated and nontreated areas of each disk, for any given concentration and substrate (Fig. 5). We also compared the total number of eggs deposited during the experiment, irrespective of position on the disk. On glass, females laid slightly fewer eggs in the presence of 20E than on control disks (Fig. 5); on paper, there were more eggs on disks treated with high concentrations of 20E (MW: 20E 10^{-2} M/control vs. control/control, P=0.015) than with 10^{-4} M 20E. On nylon, the total number of eggs laid is lower in 20E than the control assays, especially at 10^{-4} M (MW: 20E 10^{-4} M/control vs. control/control: P=0.009). No significant difference in the number of egg masses was noted (data not shown).

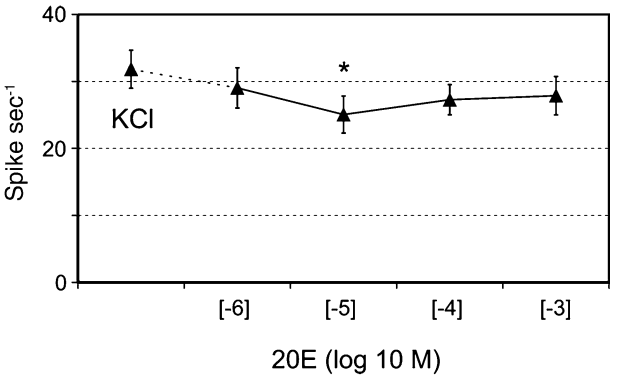


Fig. 4 Electrophysiological responses of sensilla chaetica type CRa on the ovipositor of *Ostrinia nubilalis* to ascending concentrations of 20E (from 10^{-6} to 10^{-3} M). Each *data point* represents the average of action potentials for 2 sec (mean± s.e.m.; N=16–26 recordings). Mann–Whitney test: KCl vs. 20E, *asterisk*, P<0.05

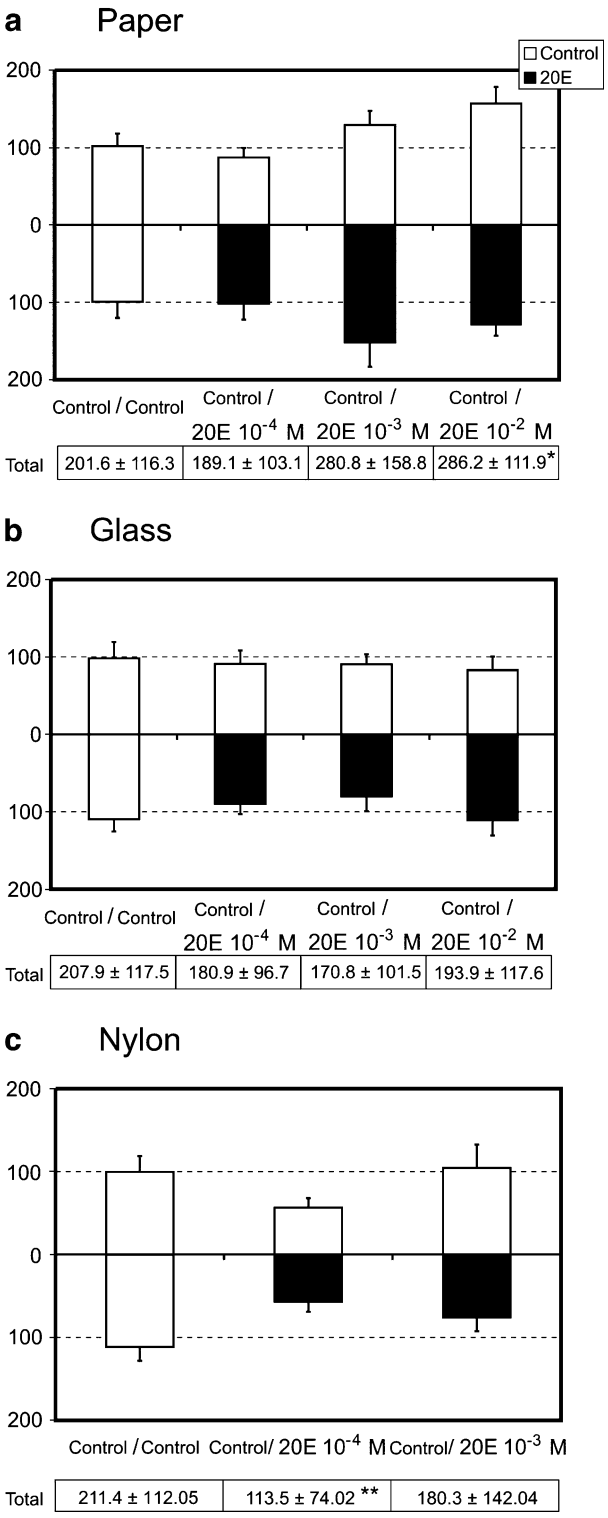


Fig. 5 Oviposition tests on artificial substrates: In choice tests, no significant differences could be observed between the treated and the nontreated areas. Different type of substrates: **a** paper, **b** glass, and **c** nylon. *White bars*: Control (solvent alone); *black bars*: 20E treatment (from 10^{-4} to 10^{-2} M). *Bars* indicate the number of eggs (mean±s.e.m.) laid on treated and nontreated parts. Under each experiment, the total number of eggs laid on the disk (mean±s.e.m.); Mann–Whitney test: control/control vs. 20E/control, *asterisk*, $P<0.05$, *double asterisk*, $P<0.01$. $N=10–14$ (with five females per test). Test duration: 1 hr

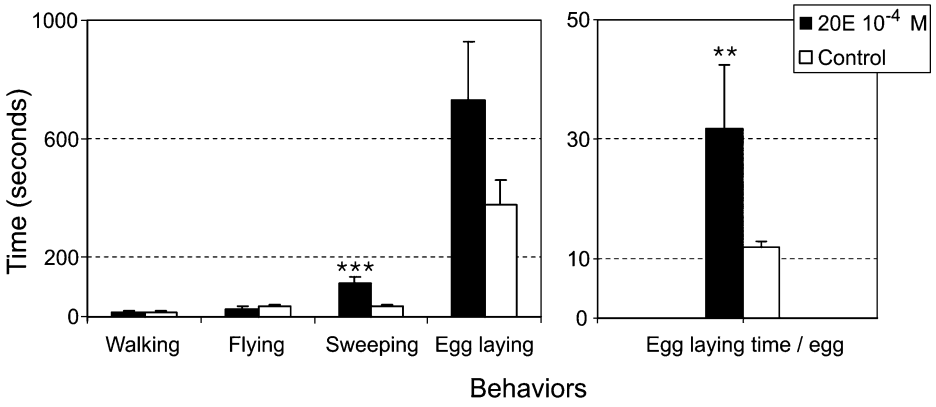


Fig. 6 Oviposition-related behaviors: Female *Ostrinia nubilalis* take longer to lay eggs on glass treated with 20E. *White bars*: Glass Petri dish treated with solvent (control); *black bars*: glass Petri dish treated with 20E 10^{-4} M (diluted in ethanol). *Bars* indicate the time in seconds (mean±s.e.m.) spent by a female for each behavior. N=10 for each treatment. Wilcoxon test: 20E vs. control, *double asterisk*, P<0.01, *triple asterisk*, P<0.001

Oviposition-related Behaviors Most females were initially resting on the side of the cage, before they started to walk or fly. There may be several flights, none exceeding a few seconds because of the size of the cage, before a female landed near the oviposition site. There were no significant difference in walking or flight between the treated and control assays (Fig. 6). Antennal movement occurred just before the flight, as well as during the

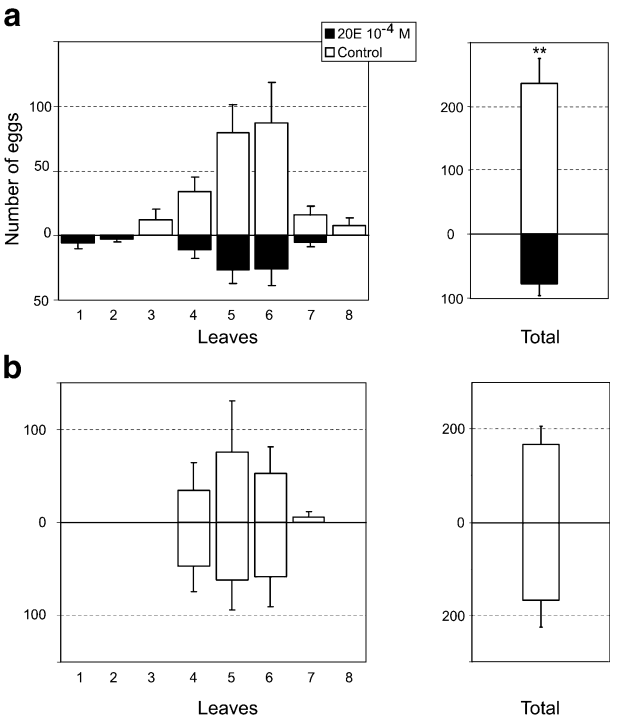


Fig. 7 Oviposition tests on plants: Female *Ostrinia nubilalis* laid significantly fewer eggs on plants treated with 20E. **a** 20E vs. control; **b** control vs. control. *White bars*: Plant treated with ultra pure water; *black bars*: plant treated with 20E 10^{-4} M (diluted in water). *Bars* indicate the number of eggs (mean±s.e.m.) laid on each leaf (1 to 8). Wilcoxon test: control vs. control and 20E vs. control, *double asterisk*, P<0.01. N=10 (20E/control), N=5 (control/control) with five females per test. Test duration: 2 hr

exploration of the substrate, but not during oviposition. The female curved her abdomen and made lateral sweeps of the substrate surface with the expanded lobes of her extruded ovipositor, which lasted significantly longer on treated than control sites (113±22.7 vs. 36.5±3.5 sec; $N=10$; $P<0.001$; Fig. 6). The female immobilized her ovipositor when she started laying eggs, one after another, in consecutive rows. While the total time to lay an egg mass took longer on the 20E substrate than on the control, the difference was not significant, although the time taken to lay each egg was (31.8±10.5 vs. 11.8±1.0 sec; $P<0.01$; Fig. 6).

Oviposition Tests on Plants The total number of eggs deposited in both experiments was comparable (20E vs. control: 315.3±55.8; control vs. control: 333.6±96.0). However, females laid significantly fewer eggs on the treated than on control plants (78.8±17.0 vs. 236.5±38.8; $P<0.01$, $N=10$) and laid more eggs on all nontreated leaves except for positions 1 and 2 (Fig. 7a). For both treatments, females laid more eggs on leaves 5 and 6. For control tests (including two plants sprayed with ultrapure water), females laid similar numbers of eggs on each plant (plant a=167±38.3; plant b=166.6±57.6 eggs), and they only laid eggs on leaves 4 to 7 with a higher number of eggs on leaves 4, 5, and 6 (Fig. 7b). This is in contrast with the distribution in the 20E vs. control, where eggs were laid on leaves 3–8 of the control plant.

Discussion

Females possess taste receptor neurons in CRb sensilla on the ventral part of the distal tarsomere, which respond to 20E, at a threshold similar to that observed in larval receptor neurons (Darazy-Choubaya 2002; Marion-Poll and Descoins 2002). One noticeable difference is that the adult female CRb sensilla have a maximal firing rate at about 50 spikes/s at 10^{-2} M 20E compared with 100–150 spikes/s for the sensilla styloconica of larvae (Marion-Poll and Descoins 2002). Such a difference was already reported between adults and larvae of *L. botrana* (Calas et al. 2006).

We did not detect any sensilla that respond to 20E on the ovipositor, but these findings should be viewed with caution, especially given the increased ovipositor-sweeping times that we observed on 20E-treated glass. We could not sample all sensilla on the ovipositor of the same insect and thus may have missed some sensilla that are responsive to 20E. Furthermore, the spikes obtained from the ovipositor sensilla were too similar in amplitude and shape to sort them accurately.

Females not only have receptors that detect 20E, they also modify their egg laying when the PE is present. However, although ECB females spent more time sweeping and took longer to lay each egg on artificial substrates treated with 20E, the total number of eggs laid on treated and control sites did not differ. In contrast, when the choice assays were carried out by using corn, females clearly avoided ovipositing on treated plants. Furthermore, the actual distribution of eggs on the leaves of treated and untreated plants varied. Preferences for different leaves have been noted in the field, largely related to the available leaf surface area (Spangler and Calvin 2000, 2001). Females laid eggs on leaves 1 and 2 of treated plants, which may be due to difficulties effectively spraying such small leaves. This possibility is supported by the fact that egg masses on treated plants were often at the tips of leaves, which are also difficult to spray. However, the overall results of this study are consistent with our initial hypothesis that ECB females, like larvae, detect and avoid sites with PEs.

It is not clear why ECB females did not avoid ovipositing on artificial substrates treated with 20E. It is possible that the quantities of 20E we used (0.014–14.40 μg/cm^2) were too low to reduce oviposition in the ECB. However, we do not think this is the case. A leaf from a plant like spinach may contain 10–40 μg/cm^2 of 20E (Lafont, personal communication). It is generally believed that much lower concentrations of 20E occur on the surface relative to the total leaf content, given the hydrophobic nature of the cuticle and the absence of an active transport system (Vrieling and Derridj 2003; Müller and Riederer 2005).

Several alternate, not mutually exclusive, explanations exist. The plant cuticle plays a major role in many plant–insect interactions (Espelie et al. 1991; Eigenbrode and Espelie 1995; Eigenbrode 2004; Müller and Riederer 2005), and epicuticular waxes may provide important cues for herbivorous insects, including ECB (Udayagiri and Mason 1997) and other Crambidae (Li and Ishikawa 2006). Some nonvolatile secondary plant compounds such as the cucurbitacins that prevent oviposition by ECB and the beet armyworm, *Spodoptera exigua* (Tallamy et al. 1997), or toosendanin that is avoided by the cabbage looper, *Trichoplusia ni* (Akhtar and Isman 2003) diffuse onto the plant surface and become associated with the waxes (Müller and Riederer 2005). In some cases, synergy of secondary compounds with the epicuticular waxes may occur, and such synergistic interactions enhance the oviposition behavior of the diamond back moth, *Plutella xylostella* (Spencer 1996; Spencer et al. 1999). Therefore, it is possible that plant epicuticular waxes could play a role in the detection of 20E.

Many insects obtain information about plants by probing the leaf surface with their antennae or tarsi before ovipositing (Chapman and Bernays 1989), so a second possibility is that ECB females only integrate information about 20E when it is detected simultaneously with other contact or volatile host plant chemicals (Bruce et al. 2005). For example, *Papilio polyxenes* females laid more eggs on artificial plants treated with a combination of contact stimulants and volatiles than on plants treated only with contact stimulants (Feeny et al. 1989). Similarly, host acceptance in the cabbage root fly, *Delia radicum*, appears to result from a synergistic response to simultaneous olfactory and contact chemo-stimulation (de Jong and Städler 1999). Females might also need to detect primary metabolites, such as soluble sugars or sugar-alcohols on the plant surface, that stimulate oviposition in the ECB and other moths species (Derridj et al. 1986; Lombarkia and Derridj 2002).

Finally, we cannot rule out that the application of 20E affects the physiology of the plant. However, if this were the case, then the effect would have to be rapid because our experiments were completed within 2 hr after treatment. 20E could be taken up and transformed by the plant into a new, more potent molecule. Maize does not produce detectable nonconjugated PEs but is capable of forming polyphosphate ecdysteroids conjugates from precursors like mevalonic acid and cholesterol (Devarenne et al. 1995; Dinan 1998). These conjugates of 20E might be more potent on the sensory system than 20E or could interact with the plant brassinosteroid receptors, which are involved in the physiological reactions of plants to phyto-aggressors or stress (Krishna 2003; Nakashita et al. 2003; Wang and He 2004).

Additional experiments must be carried out to test the hypotheses presented to explain why the presence of 20E on artificial substrates does not deter oviposition by the ECB but does on intact plants. These will include assays that look at oviposition on artificial substrates to which primary metabolites or maize odors have been added. In addition, it will require the analysis of the leaf surface compounds before and after applying 20E, together with an analysis of volatile compounds released to check if the chemicals accessible to female ECB are modified by 20E. It would also be interesting to investigate why 20E on artificial substrates affects oviposition by grape berry moth, *L. botrana*, females (Calas et al.

2006) but not in the case of the ECB. Is this related to the greater variability of cues that a polyphagous species encounters when selecting suitable host plants compared with a monophagous one?

Acknowledgments We thank René Lafont (University Paris VI) for his gift of 20E used in this study and numerous discussions. We thank Alan Cork for reviewing the manuscript as well as two anonymous referees and the editor for their useful suggestions. This work was supported by an INCO-DEV program SUSVEG-ASIA.

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Keywords *Trissolcus basalis* · *Nezara viridula* · Egg parasitoid · Motivated searching · Arrestment response · Indirect host-related cue · Kairomone · Cuticular hydrocarbons · Hexane extract · Solid-phase microextraction

Introduction

Insect parasitoids are known to use semiochemicals for location and recognition of hosts (Vinson 1998), and several studies have been published on the use of host-produced kairomones by hymenopteran parasitoids (Vinson 1991, 1998; Godfray 1994; Quicke 1997; Vet 1999). The possibility of using semiochemicals to enhance the efficacy of parasitoids in biological control programs also has been investigated (Lewis and Martin 1990; Vet and Dicke 1992; Powell and Pickett 2003). In the generally accepted paradigm, parasitoids use volatile cues from the host plant complex to locate the habitat of their hosts, and then host-derived stimuli are used for host location, recognition, and acceptance over shorter distances (Vinson 1991, 1998; Vet and Dicke 1992). Host-derived stimuli can be directly emitted by the host stage, or they can be indirectly associated with the attacked host stages (Vinson 1991, 1998; Godfray 1994; Quicke 1997). The switch from cues from the host plant complex to indirect host-derived cues is normally associated with an adjustment of female behavior from a directed movement (e.g., flying from one location to another in the habitat) to a motivated searching response within and around an area contaminated by potential hosts (i.e., reduced linear speed and increase in ortho- and klinokinetic movements; Vinson 1984; 1998).

Indirect host-related cues that elicit arrestment and motivated searching in an area where there is a high probability of finding hosts are often exploited for host location by hymenopteran egg parasitoids in the families Trichogrammatidae and Scelionidae. These egg parasitoids show motivated searching upon contact with indirect host-related cues such as lepidopteran scales (Laing 1937) or pentatomid bug footprints (Colazza et al. 1999b). Motivated searching induced by scales shed by moths has been observed with several *Trichogramma* spp. and with *Telenomus busseolae* Gahan (Laing 1937; Beevers et al. 1981; Lewis et al. 1982; Gardner and van Lenteren 1986; Colazza and Rosi 2001), whereas motivated searching induced by chemical residues left on the substrate by adult pentatomid bugs has been observed with several *Trissolcus* spp. and with *Telenomus podisi* (Ashmed) (Colazza et al. 1999b; Conti et al. 2003, 2004; Borges et al. 2003; Salerno et al. 2006). The motivated searching behavior of egg parasitoids to these types of cues is characterized by an initial prolonged motionless period with the antennae kept in contact with the surface (arrestment response). Then the wasps, while drumming the surface with their antennae, exhibit intense searching behavior of the surrounding area, characterized by variation in orthokinetic and klinotaxic locomotion, and increased turning frequency. In this way, wasps are kept in or around the contaminated area, with the response reinforced by systematic returns to the stimulus after losing contact with it. If no host eggs are found, searching behavior slowly decreases, and the normal walking pattern is gradually resumed (Laing 1937; Gardner and van Lenteren 1986; Colazza et al. 1999b; Conti et al. 2003, 2004; Peri et al. 2006).

Previous papers have demonstrated the importance of chemical residues left by host *Nezara viridula* (L.) (Heteroptera: Pentatomidae) adults in the host location behavior of females of the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae). Wasp females were found to have an innate response to host chemical residues, with a strong preference for residues left by *N. viridula* females rather than males (Colazza et al. 1999b; Peri et al. 2006). Furthermore, experience gained during foraging on host chemical

residues can strongly affect a wasp's motivated searching pattern (Peri et al. 2006). The role of host residues in relation to the host specificity of *T. basalis* also has been demonstrated (Salerno et al. 2006).

In this research, the source and the nature of the chemical residues deposited by *N. viridula* adults that induce arrestment responses from *T. basalis* females were investigated. It was found that the hydrocarbons present in the wax layer of *N. viridula* cuticle act as contact kairomones, causing arrestment of female wasps. A homologous series of straight-chain hydrocarbons was identified and quantified in solvent and solid-phase micro-extraction (SPME) extracts of host cuticle. Because the cuticular hydrocarbon profiles of *N. viridula* adults showed qualitative and quantitative sex-specific differences, the roles of specific hydrocarbons in the wasp's arrestment response were examined in behavioral bioassays. A reconstructed blend of straight-chain cuticular hydrocarbons induced arrestment responses by wasp females, and nonadecane (nC_{19}), a male-specific hydrocarbon, was used by *T. basalis* females to discriminate between chemical residues left by host females and host males.

Methods and Materials

Maintenance of Insect Colonies *Nezara viridula* and *T. basalis* colonies were established and reinvigorated regularly with insects collected from fields located around Palermo, Italy. Both colonies were reared in an environmentally controlled room at 25±1°C, 70±10 % R. H., and photoperiod of 16L:8D. The *N. viridula* colony was reared in wooden cages (25× 25×40 cm height) with two 5-cm diam mesh-covered holes for ventilation, and colonies were fed with seasonal fresh vegetables. The colony of *T. basalis* was reared in 16-ml glass tubes and fed with a 10% honey–water solution. Egg masses of *N. viridula* were collected daily and used to maintain both colonies. Single *N. viridula* egg masses were exposed to three–four female wasps and then held in the same environmental conditions until adult emergence. Every 2–3 d, newly emerged *N. viridula* adults were isolated in different rearing cages so that individuals of known age were continuously available for experiments.

Stimulus Preparation Procedure Mated *N. viridula* adults were used in all bioassays. Males were 4- to 8-d-old, whereas females were 2–4 d older to ensure that they were in the pre-ovipositional physiological state; our previous work had shown that *T. basalis* responded strongly to residues associated with mated, gravid host females (Colazza et al. 1999b). Dissected adult body parts, hexane extracts of insect cuticle, and reconstructed blends of synthetic hydrocarbons used for bioassays were prepared as follows:

(1) *Tarsi and Scutella Dissected from Males and Females of N. viridula*. Responses of *T. basalis* females to tarsi and scutella dissected from *N. viridula* adults were tested in arena bioassays. The scutellum was selected as a control body part because it should not be contaminated by compounds from *N. viridula* legs during grooming behaviors. *Nezara viridula* adults were killed by freezing at −18°C for about 1 hr, and dissected under a binocular stereo microscope. Fine scissors were used to clip off the legs at the coxa level, and to cut the scutellum from the pronotum. Dissected legs and scutella were used a few minutes after dissections for bioassays.

(2) *Deproteinized N. viridula Tarsi*. Legs used for the experiment were dissected from adults as described above. The left legs were treated with 1% trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) (Sigma, St. Louis, MO, USA) buffered with Hank's

balanced salt solution (HBSS; pH 7.4), whereas the right legs were treated with HBSS only and used as a control. Both sets of legs were incubated at 37°C for 80 min, then rinsed with distilled H_2O, air-dried at room temperature, and used for bioassays.

(3) *Hexane Extracts of N. viridula Cuticular Lipids*. Frozen adults were dissected into legs, wings, pronotum, and scutellum as described above. The inside of the pronotum cuticle was cleaned as thoroughly as possible to remove traces of adhering tissues. The mesonotum, metanotum, and abdomen were not used in experiments because of possible contamination by defensive chemicals from the glandular structures in these body sections, which can induce arrestment of *T. basalis* females (Mattiacci et al. 1993). Dissected body parts from three males and three females were weighed, then placed in 15-ml glass vials and extracted with 10 ml hexane at 30°C for 2 hr. The amount of cuticle obtained (mean±SD) from dissected body parts of three adults was 24±1 mg for males, and 32±3 mg for females. After removal of the body parts, the resulting extracts were evaporated under a gentle nitrogen stream, and redissolved in 1 ml hexane. Extracts were prepared from five groups of three bugs each, and after each extract was checked by GC (see below), the extracts were pooled by gender, and stored at −18°C until assayed.

(4) *Reconstructed Blend of Host Female Straight-Chain Cuticular Hydrocarbons*. A blend of straight-chain alkanes of *N. viridula* females was reconstructed, normalizing the concentrations of the alkanes found in the crude extract (see Table 1) to nonacosane, the most abundant compound. Two stock hydrocarbon blends were prepared as hexane solutions. The first stock solution contained (in mg/l): nC$_{29}$ (1,000), nC$_{26}$ (910), nC$_{27}$ (800), nC$_{31}$ (760), nC$_{28}$ (670), nC$_{25}$ (500), nC$_{24}$ (450), nC$_{30}$ (420), nC$_{23}$ (270), nC$_{32}$ (250), nC$_{33}$ (210), and nC$_{22}$ (190). The second stock solution contained (in mg/l) nC$_{21}$ (700), nC$_{34}$ (500), and nC$_{20}$ (200). The working solution of the total reconstructed blend then was obtained by combining 10 ml of the first stock solution and 1 ml of the second stock solution and diluting it to 100 ml. All hydrocarbons were analytical grade GC standards (Fluka). The reconstructed blends were stored at −18°C until assayed.

(5) *Hexane Extracts of N. viridula Female Blended with Synthetic Nonadecane* (nC$_{19}$). Dissected body parts from 12 *N. viridula* females (155 mg of cuticle) were cut up and divided in four portions, and each portion was extracted in 10 ml of hexane, evaporated under a stream of nitrogen, and redissolved in hexane to a total volume of 1 ml as described above. Then, three of the four extracts were spiked with synthetic nC$_{19}$ in hexane to provide final concentrations of 0.1, 1.0, and 10.0 μg nC$_{19}$ per ml in the spiked extracts. The four extracts were stored at −18°C until assayed.

Open Arena Bioassay Procedure Female *T. basalis* used for bioassays were held with males for 24 hr to ensure that they were mated, then females were individually isolated in small vials with a drop of honey-water and used in bioassays when 2 to 3 d old. Thus, all wasp females were naïve with respect to both oviposition experience and contact with chemical cues produced by their *N. viridula* hosts. Female wasps were allowed to acclimate in the bioassay room for at least 1 hr before bioassays. Bioassays with *T. basalis* females were conducted in open arenas consisting of rectangular filter paper sheets (20×20 cm). In the center of the arena, a 4-cm-diam circle (12.56 cm^2, about 3% of the entire arena), defined by a still image of the arena taken with the video tracking and motion analysis software (see below) so that the arena remained free of any marks, was treated with test

stimuli. The rest of the open arena was left uncontaminated. Each arena was used for testing five wasp females (previous studies had shown that test wasps were unaffected by any residues from previously tested wasps; Colazza et al. 1999b; Peri et al. 2006). For each experiment, the following bioassay procedure was used.

(1) *Tarsi and Scutella Dissected from Males and Females of N. viridula*. Tarsi and scutella, dissected from four males and four females of *N. viridula*, were gently wiped on the test areas of the open arenas with fine forceps. Eighty wasps were tested individually.
(2) *Deproteinized N. viridula Legs.* Legs from six *N. viridula* adults (three males and three females), deproteinized by treatment with trypsin-EDTA or treated with buffer only, were wiped on the treatment areas of the arenas. The experiment was repeated three times for a total of 60 tested wasps.
(3) *Hexane Extracts of N. viridula Cuticular Lipids*. Hexane extracts of male and female *N. viridula* cuticular hydrocarbons were tested at doses of 3×10^{-3}, 1, and 10 dissected adult equivalent (DAE) by pipetting 1, 333, and 3,333 μl of extracts onto the treatment area. Before using the 3×10^{-3} DAE dosage, the aliquot of 1 μl of solution was diluted in 200 μl of hexane to allow even distribution over the entire treated area. The 10 DAE dosage was applied by repetitive pipetting of about 50 μl of solution, allowing the hexane to evaporate between repetitions. For each dosage and for both *N. viridula* genders, 15 *T. basalis* females were tested for a total of 180 wasps.
(4) *Reconstructed Host Female Blend of Straight-chain CHs*. One DAE of the reconstructed hydrocarbon blend was tested by pipetting 40 μl of a hexane solution containing 0.1 mg/ml of the most abundant component (nC$_{29}$) and proportional amounts of the minor components onto the treatment areas. The bioassay was repeated four times with a total of 20 female wasps.
(5) *Hexane Extracts of Female N. viridula, Spiked with Synthetic* nC$_{19}$. Aliquots of extract containing 1 DAE of the crude extract spiked with four different concentrations of nC$_{19}$ were tested by pipetting 333 μl of a solution onto the treatment area of an open arena for each of the different solutions. The experiment was repeated three times for a total of 60 tested wasps.

For experiments 3, 4, and 5, stimuli were applied as drops, and therefore the chemicals on the treated areas were patchily distributed. Wasp females were gently released singly from a small vial (2 ml) onto the center of the treated area. Wasp behavior was scored as "response" when the female, immediately after being released, remained unmoving with the antennae held in contact with the arena surface (arrestment response). Females that did not show the arrestment response after touching the treated area with their antennae were recaptured with the same vial, and retested after ~1 min. Wasps that did not show the arrestment response after three trials were scored as "non-response". After the initial arrestment response, responding females typically started to walk slowly with many turns while drumming the arena surface with their antennae, stopping again each time they came back to the treated area (motivated searching behavior). Wasps' movements were recorded with a video tracking and motion analysis system (see below) until they flew away from or walked off the arena. The time spent on the entire arena was scored as the arena residence time. This value was used, rather than only recording the time spent on the treated area, because we felt that the time spent by wasp females on the entire arena was more representative of the active searching behaviors stimulated by the kairomonal stimuli.

Wasps' walking patterns were recorded with a CCD camera (Sony M370) equipped with a zoom lens and mounted above the center of the arena. The video camera was connected to a video monitor (Sony PVM 2130QM) and a desktop PC equipped with a video frame grabber (Miro PC-TV—Pinnacle Systems, Mountain View, CA, USA). A composite video signal from the camera was fed into custom video tracking and motion analysis software developed for the Linux operating system (Colazza et al. 1999a). A threshold value of 1 mm resolution was used during recordings to minimize creation of artifacts because of noise in the system. Different treatments among different stimuli were tested at the same time, and stimuli were tested on several different days. All experiments were carried out from 1000–1500 hours, in an isolated room at 26±1°C, with the arena illuminated by two 18-cm-long fluorescent lights (full spectrum 5,900 K, 11 W; Lival, Italy).

Analysis of N. viridula Cuticular Hydrocarbons Hexane extracts of *N. viridula* cuticle and body parts were analyzed by gas chromatography (GC) with a Hewlett-Packard 6890 GC (Avondale, PA, USA) with a flame ionization detector (FID), and equipped with an HP5-MS column (30 m×0.2 mm, 0.25-μm film, J&W Scientific, Folsom, CA, USA). A 4-mm ID straight glass injector liner packed with glass wool was used, with the injector temperature at 250°C. Injections (1 μl) were made in splitless mode with helium as carrier gas (1.5 ml/min). The oven temperature program was 150°C for 2 min, then 5°C/min to 280°C, and hold for 20 min. The detector temperature was 300°C. A mixture of *n*-alkanes (nC$_{19}$–nC$_{36}$) (all 99% purity; Fluka) in hexane served as external standards to identify and quantify each compound.

Solid-phase Microextraction of Cuticular Hydrocarbons and Residues Left by N. viridula Adults Direct-contact solid-phase microextraction (DC-SPME) was used to collect *N. viridula* cuticular hydrocarbons and the chemical residues left by *N. viridula* walking on a glass plate (10×10 cm). A 100-μm film thickness polydimethylsiloxane SPME fiber was used (Supelco Inc., Bellefonte, PA, USA), conditioning it before use at 250°C for 30 min in a GC injector port. *Nezara viridula* cuticular hydrocarbons were extracted from freeze-killed adults by gently wiping the SPME fiber over the head, pronotum, and legs for 60 sec. The same fiber was used to sample 10 individuals of the same gender before being desorbed in the GC inlet port. Residues left by *N. viridula* were sampled by wiping the SPME fiber over a glass plate upon which an adult bug had been confined for 1 hr. The same fiber was used to sample the residues left by three adults of the same gender before desorbing the fiber. Samples were analyzed by desorbing the loaded SPME fiber in the GC injector port (270°C) for 2 min, using a 0.75-mm ID glass injector liner in splitless mode, with the same column and temperature program as described above.

Precision and Detection Limits The method linearity of solvent extractions was determined for each identified cuticular *n*-alkane at five concentrations ranging from 1 to 30 ng μl^{-1}, and for each calibration curve the regression coefficient (R^2) was determined. Linearity was considered achieved when R^2 was 0.995. The calibration curves were then used to quantify the amount of each *n*-alkane in the extracts. The limit of quantification (LOQ) was fixed using a solution of hydrocarbon standards at a concentration of 0.5 ng μl^{-1} per compound. The limit of detection (LOD) for each analysis was defined by measuring progressively more dilute concentrations of the identified hydrocarbons until a signal-to-noise ratio of 3:1 was reached. The relative standard deviation (RSD) for each compound was determined.

Statistical Analyses Data sets were first checked for assumptions of normality with a Shapiro-Wilks test at $P=0.05$. The numbers of responding female wasps were analyzed by χ^2 tests for independence in a two-way contingency table. Arena residence times were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc test for multiple comparisons between means. Contrast analyses between naïve and experienced female wasps from the third experiment were done using *t* tests. All statistical analyses were done using Statistica 6.0 for Windows (StatSoft Inc., Tulsa, OK, USA).

Results

Open Arena Bioassay. Tarsi and Scutellum Dissected from Males and Females of N. viridula Every female *T. basalis* tested (100% of 80 trials) showed arrestment responses when exposed to arenas treated by rubbing with tarsi or scutella dissected from *N. viridula* adults. The wasps' arena residence times were significantly affected by host gender, but not by the two different dissected body parts ($F_{3,\ 76}=8.55$, $P<0.001$, one-way ANOVA) (Fig. 1). Wasps stayed longer on the arenas treated with tarsi and scutella dissected from female hosts than on arenas treated with the analogous dissected body parts from male hosts

Table 1 Straight-chain cuticular hydrocarbons extracted from three female and three male *N. viridula* by hexane extractions of dissected body parts, by DC-SPME of freeze-killed insects, and by DC-SPME of chemical residues left by *N. viridula* adults walking on glass plates

Peak #	Ret. time	Linear hydrocarbon	Hexane extracts of dissected body parts		Student *t*-test	DC-SPME samples			
			ng/mg cuticle ± SD $N=5$			Whole body		Chemical residues	
			♀♀	♂♂		♀♀	♂♂	♀♀	♂♂
1	11.51	nC$_{19}$	-	66±15		-	+	-	+
2	13.29	nC$_{20}$	9.8±0.8	9±1	ns	-	-	-	-
3	15.04	nC$_{21}$	33±3	26±4	ns	-	-	-	-
4	16.75	nC$_{22}$	88±7	64±8	ns	-	-	-	-
5	18.41	nC$_{23}$	192±16	130±13	*	-	-	-	-
6	20.01	nC$_{24}$	348±39	215±16	*	+	+	+	+
7	21.56	nC$_{25}$	425±62	238±13	*	+	+	+	+
8	23.04	nC$_{26}$	434±77	221±10	ns	+	+	+	+
9	24.50	nC$_{27}$	381±81	173±7	ns	+	+	+	+
10	25.88	nC$_{28}$	318±73	135±6	ns	+	+	+	+
11	27.22	nC$_{29}$	477±99	222±27	ns	+	+	+	+
12	28.54	nC$_{30}$	201±51	81±1	ns	+	+	+	+
13	30.04	nC$_{31}$	363±87	141±4	ns	+	+	+	+
14	31.80	nC$_{32}$	118±36	37±3	ns	-	-	-	-
14	33.90	nC$_{33}$	99±27	36±3	ns	-	-	-	-
16	36.48	nC$_{34}$	26±8	11±2	ns	-	-	-	-

Peak numbers correspond to Figs. 4 and 5.

Data represent means ± SD.

* = $P\leq0.05$; ns=not significant, - = not detected, + = detected.

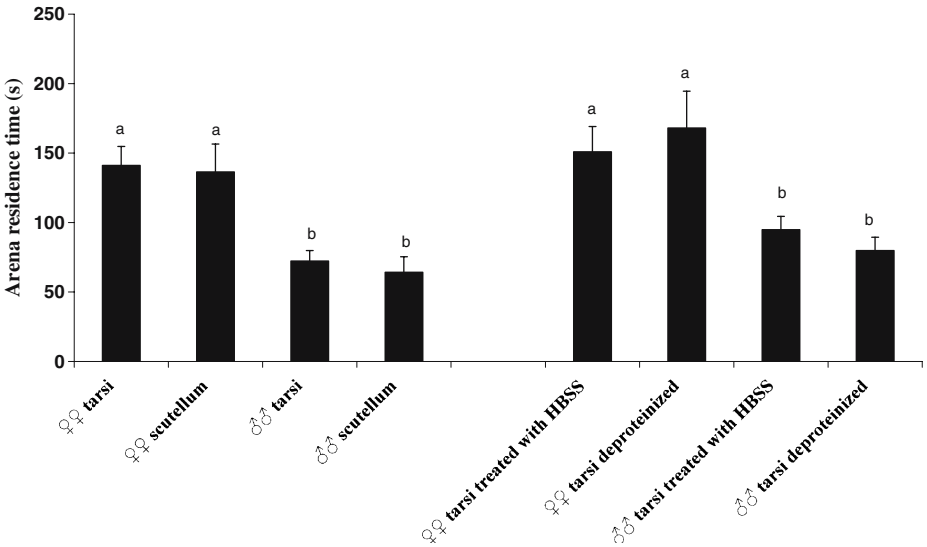


Fig. 1 Arena residence times (sec) of *T. basalis* females tested on arenas on which dissected body parts (tarsi and scutella) from females and males of *N. viridula* had been rubbed. Tarsi and scutella were tested untreated. Tarsi were also tested after they had been deproteinized by treatment with trypsin-EDTA buffered with Hank's balanced salt solution (HBSS), and with controls treated with only HBSS. Bars indicate mean ± SE. Different letters above the bars indicate significantly different means (ANOVA, Tukey HSD)

(Fig. 1). There were no differences in *T. basalis* arena residence times when the arenas were treated with tarsi or scutella dissected from adults of the same gender (Fig. 1).

Deproteinized N. viridula Tarsi Every female wasp (100% of 60 trials) responded to arenas rubbed with host legs deproteinized by treatment with trypsin, or with buffer-treated controls. Consistent with previous results, arena residence times of wasps were influenced by host gender ($F_{3,\ 56}$=5.94, P=0.001, one-way ANOVA; Fig. 1), whereas no differences were observed between the two treatments.

Hexane Extracts of N. viridula Cuticular Lipids Wasps' responses to hexane extracts of *N. viridula* cuticular lipids were significantly affected by dose (expressed as dissected adult equivalent, DAE) applied to test arenas (Fig. 2). Similar percentages of wasps responded to arenas treated with 3×10^{-3} DAE or 1 DAE (χ^2=1.7, P=0.19; χ^2=3.75, P=0.052; for male and female extracts, respectively; Fig. 2). However, fewer wasps responded to arenas treated with 10 DAE compared to arenas treated with 1 DAE (χ^2=10.8, P=0.001; χ^2=5.96, P=0.01; for male and female extracts, respectively; Fig. 2). Wasps that responded to the cuticular extracts showed similar arena residence times ($F_{2,\ 58}$=1.33, P=0.27; ANOVA; $F_{2,\ 65}$=0.4, P=0.70; for male and female extracts, respectively, one-way ANOVA). Similar to previous results, *T. basalis* females spent more time on arenas treated with hexane extracts of females than on arenas treated with extracts of males ($F_{1,\ 127}$=16.32, P<0.001; ANOVA).

Reconstructed Blend of Straight-chain Cuticular Hydrocarbons Most of the wasp females (80% over 20 trials) responded to arenas treated with a reconstructed blend of straight-chain

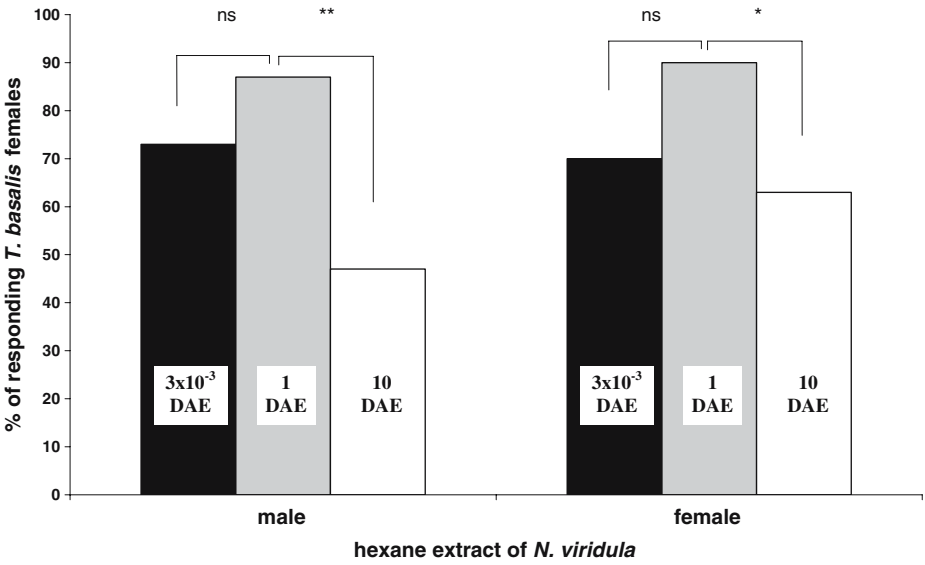


Fig. 2 Percentage of *T. basalis* females that showed arrestment responses to hexane extracts of male and female *N. viridula* applied onto arenas at doses of 3×10^{-3}, 1, and 10 dissected adult equivalents (DAE). Data were compared by χ^2 tests for independence. ns=not significant; *$P\leq0.05$

cuticular hydrocarbons, but females spent less time in these arenas than on arenas treated with crude extracts of *N. viridula* females (209±112 vs. 101±47 sec, for crude extract of *N. viridula* females and reconstructed blend of straight-chain CHs, respectively; $t=3.55$, $df=29$, $P=0.001$, t test).

Hexane Extract of N. viridula Females Spiked with nC$_{19}$ Wasps' arrestment behaviors toward hexane extracts of *N. viridula* females were influenced by the addition of synthetic nC$_{19}$ ($F_{3,\ 54}=4.68$, $P=0.005$, one-way ANOVA; Fig. 3). Every *T. basalis* female (100% of 15 trials) responded to 1 DAE of the crude extract and to 1 DAE of the crude extract spiked with 0.033 and 0.33 μg nC$_{19}$, and their arena residence times were not significantly different (Fig. 3). However, when 1 DAE of the crude extracts was treated with 3.33 μg nC$_{19}$, fewer wasps responded (87% over 15 trials), and they showed reduced arena residence times compared to wasps presented with the unspiked treatment (Fig. 3).

Chemical Analysis of N. viridula CHs Satisfactory validation data for the solvent extraction method in terms of both linearity (R^2 ranged between 0.995 and 0.998) and reproducibility (RSD% better than 20%) were achieved. The LOQs were fixed at 0.5 ng, and the LODs were established at 0.1 ng.

A homologous series of *n*-alkanes with chain lengths from 19 to 36 carbon atoms was identified and quantified in the solvent extracts of *N. viridula* (Table 1). The average amount of all cuticular hydrocarbons extracted from a single *N. viridula* female was about twice that recovered from equivalent weights of male *N. viridula* body parts (3,540±1,138 and 1,818±85 ng mg^{-1} cuticle from females ($N=5$) and males ($N=5$), respectively; $t=-2.61$; $P=0.05$, t test). The hydrocarbon profiles showed consistent sex-specific qualitative and quantitative differences. nC$_{19}$ was detected only in male extracts, where it accounted for

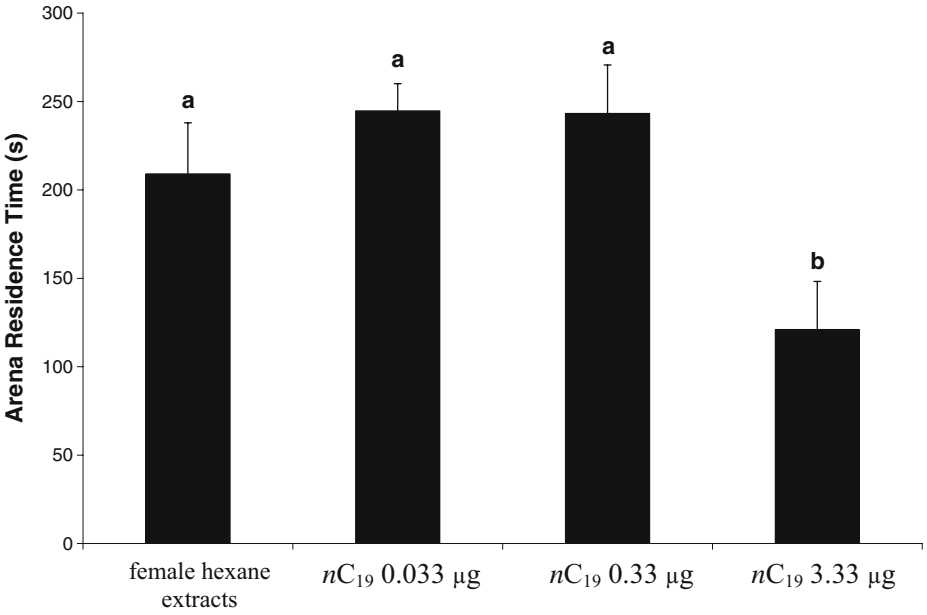


Fig. 3 Arena residence times (sec) of *T. basalis* females tested on arenas treated with aliquots of crude extracts of *N. viridula* females containing increasing concentrations of nC_{19}. For each test, 350 µl of solution was applied. Bars indicate mean±SE. Different letters above the bars indicate significantly different means (ANOVA, Tukey HSD)

3.4±1.1 % of the total hydrocarbons detected (Fig. 4, peak 1). Extracts from females contained significantly more nC_{23}, nC_{24}, and nC_{25} than extracts from males (Fig. 4, peaks 5, 6 and 7; Table 1).

Solid-phase Microextraction of Cuticular Hydrocarbons from Insect Cuticle and from Residues Left by N. viridula Adults Walking on Glass Sampling cuticular hydrocarbons from *N. viridula* adults with DC-SPME did not yield any linear hydrocarbons that were not present in the solvent extracts of *N. viridula* (Table 1). Furthermore, only about half of the total number of compounds identified from solvent extracts were also detected in the DC-SPME extracts (e.g., nC_{19}, nC_{24}, nC_{25}, nC_{26}, nC_{27}, nC_{28}, nC_{29}, nC_{30}, and nC_{31}) (Table 1; Fig. 5). When the chemical residues left by adult female and male *N. viridula* walking on a glass plate were sampled with DC-SPME, straight-chain alkanes from C_{24} to C_{31} were detected in footprints from both sexes, whereas only male footprints contained nC_{19} (Fig. 5, Table 1).

Discussion

Many insects deposit chemical residues on substrates, and these traces can provide intraspecific signals and/or interspecific cues that modify the behaviors of receiving organisms. For example, bumblebees leave residues on flowers that allow conspecifics to avoid recently visited flowers (Eltz 2006). Chemical residues can also serve as kairomones

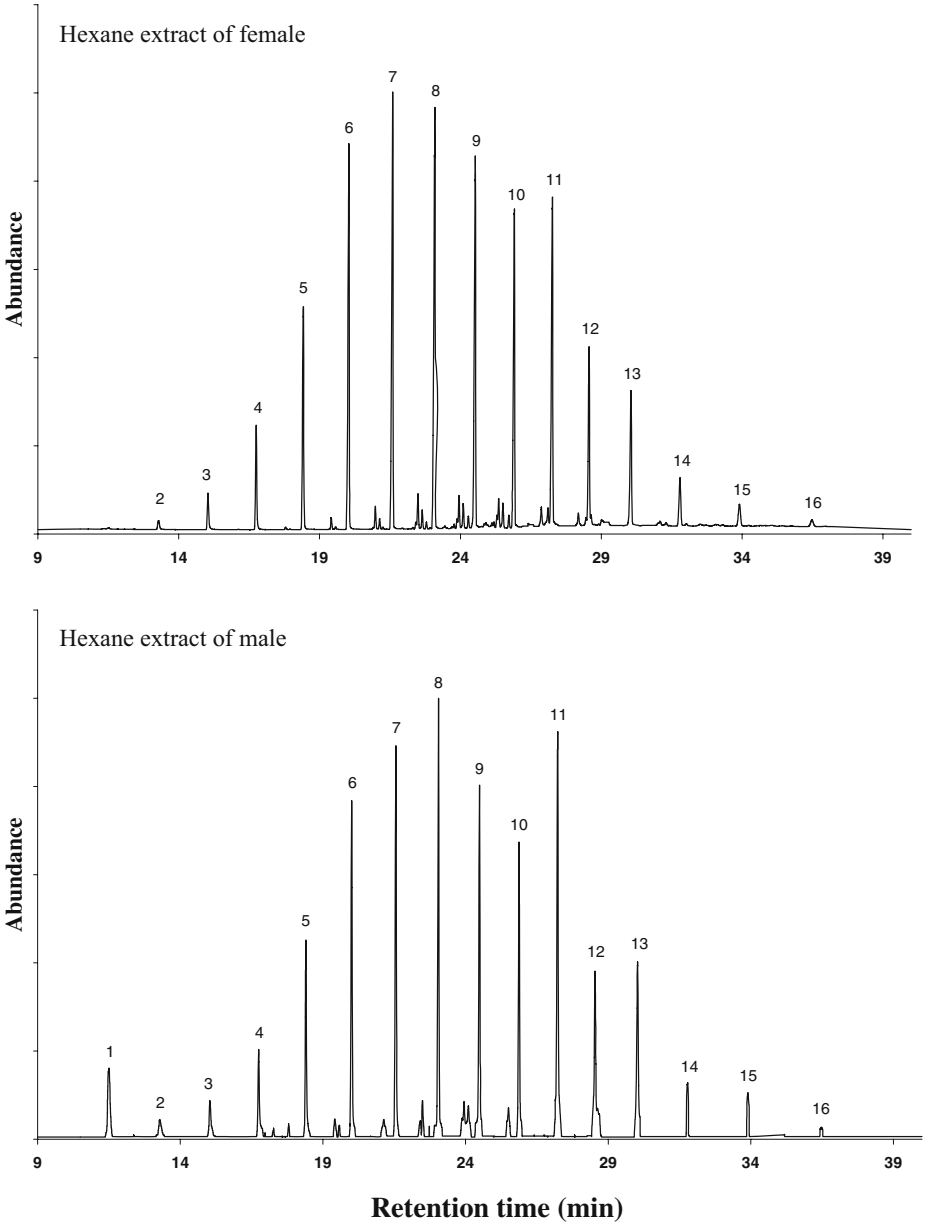


Fig. 4 Representative gas chromatograms of cuticular hydrocarbons extracted from adult female (top) and male (bottom) *N. viridula* with hexane. For peak identities, see Table 1, and "Methods and Materials" for analysis conditions

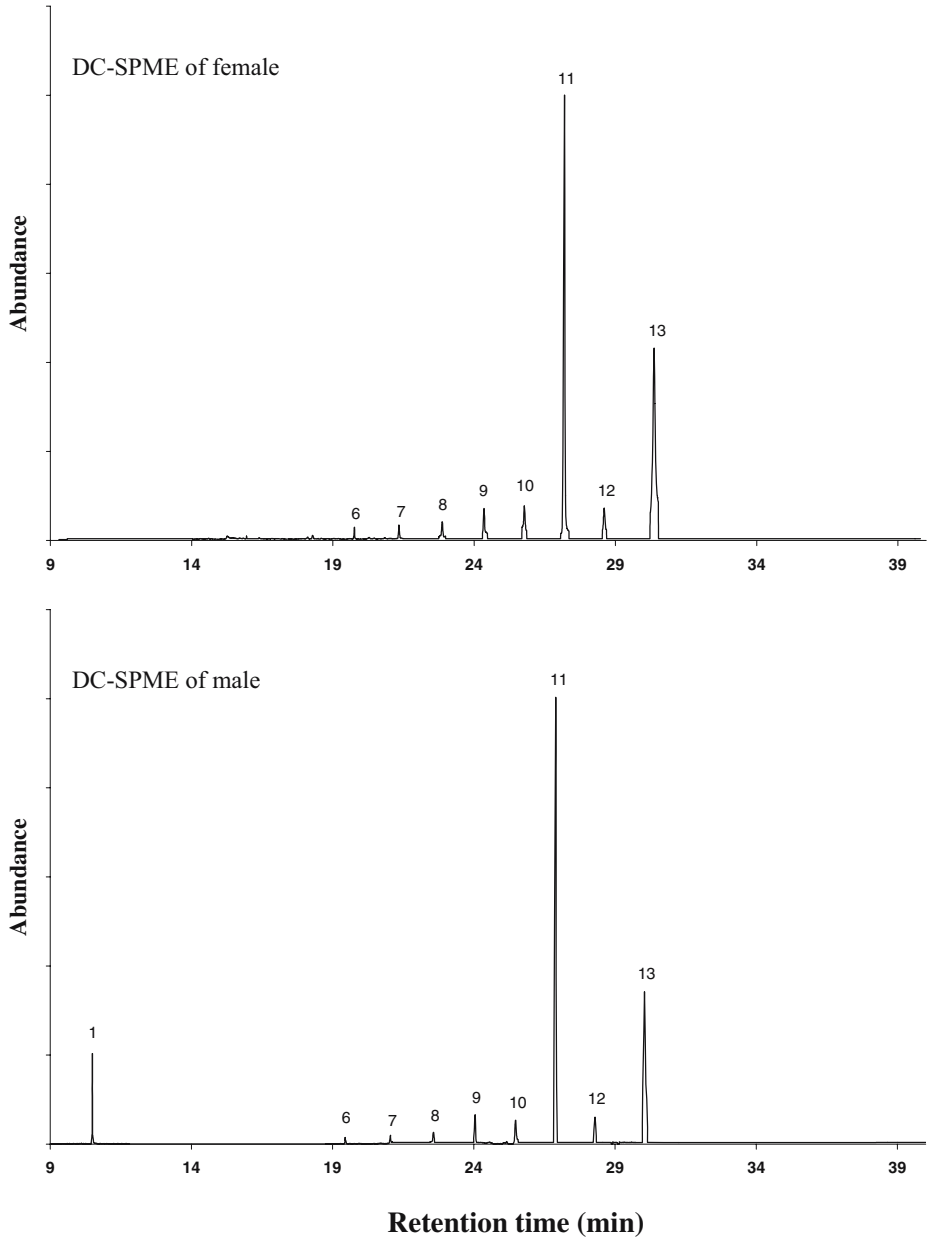


Fig. 5 Representative gas chromatograms of cuticular hydrocarbons extracted from adult female (top) and male (bottom) *N. viridula* with DC-SPME wiping the fiber over the head, pronotum, and legs. For peak identities, see Table 1, and "Methods and Materials" for analysis conditions

for natural enemies, as in the case of trails left by larvae of the pine looper moth, *Bupalus pinarius* L (Lepidoptera: Geometridae), which are exploited as cues by the larval parasitoid *Poecilostictus cothurnatus* (= *Platylabus cothurnatus*) Gravenhorst (Hymenoptera: Ichneumonidae) (Klomp 1981). Chemical residues can also be indirectly associated with the host as is the case with chemical residues left on the substrate by adult pentatomid bugs (Colazza et al. 1999b; Borges et al. 2003; Conti et al. 2003). Various origins for the chemical residues can be considered. For instance, the chemical footprints of lady beetles (Coleoptera: Coccinellidae) are secreted from the tenent hairs on the bottom of the tarsi, and the footprints consist of nonvolatile lipids identical to those in the cuticular waxes (Kosaki and Yamaoka 1996). In nature, lady beetle footprints deter oviposition by conspecifics, and deter foraging by aphid parasitoids (Hemptinne et al. 2001; Nakashima et al. 2004).

Our data showed that chemicals on the tarsi and scutella of *N. viridula* induced motivated searching responses by *T. basalis*. These results suggest that the kairomones mediating these responses are part of the wax layer of *N. viridula*, rather than being secreted by specialized glands such as those on *N. viridula* legs, which are the source of lipid adhesive fluid (Ghazi-Bayat and Hasenfuss 1980), or the tarsal glands situated in the tarsomer (R. Romani and N. Isidoro, personal communication). Whether or not the amounts of secreted cuticular hydrocarbons are more abundant in the tarsi than in the other body parts of *N. viridula* cuticle remains to be determined.

The insect wax layer contains a complex mixture of compounds that include straight-chain saturated and unsaturated hydrocarbons, and methyl-branched hydrocarbons (Gibbs 1998; Howard and Blomquist 2005). Several studies have shown that straight-chain hydrocarbons and related long-chain carboxylic acids that are present in moth scales elicit arrestment responses from foraging *Trichogramma* wasps (Boo and Yang 2000). For example, hexane extracts of adult *Scripophaga incertulas* Walker (Lepidoptera: Pyralidae) elicited searching responses from the egg parasitoid *Trichogramma japonicum* Ashmead (Rani et al. 2007), and straight-chain hydrocarbons from scales of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) induced searching by *Trichogramma evanescens* Westwood. In the latter species, *n*-tricosane (nC_{23}) was identified as the most active component of the kairomonal blend (Jones et al. 1973).

It had been shown previously that the footprints of pentatomid bugs stimulated motivated searching by scelionid egg parasitoids (Colazza et al. 1999b; Conti et al. 2004), but the chemistry of the footprints had not been investigated. The results of the present study showed that these kairomonal cues are hexane-soluble, suggesting that the cuticular hydrocarbons might be involved. However, a reconstructed blend of straight-chain hydrocarbons extracted from female *N. viridula* induced only weak arrestment responses in *T. basalis* females. Thus, other components of the wax layer of *N. viridula*, such as branched hydrocarbons or more polar components of the wax layer, must be important in the elicitation of the full response. Analyses of *N. viridula* extracts have shown the presence of mono- and dimethyl-branched alkanes, and more polar lipids, and their possible roles in the wasps' responses are under investigation.

A dose-response effect of hexane extracts from the scales of *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) has been observed with *T. evanescens* females (Schmidt and Carter 1992). The responses of female *T. basalis* were also clearly influenced by different doses of hexane extracts of adults. *Trissolcus basalis* females responded to approximately 7.3 ng/cm^2 of hydrocarbons, which represents the amount of hydrocarbons (114 ng) applied on the 12.56-cm^2 treated area of the bioassay arenas at the dosage of 3×10^{-3} DAE of *N. viridula* females. It would clearly be an advantage for parasitoid females to perceive low levels of kairomones. Conversely, the adaptive significance of the decreased

responses of *T. basalis* females when presented with the much larger dose of hydrocarbons (e.g., ~30,000 ng/cm^2, applied to the 12.56-cm^2 treatment area, corresponding to the 10-DAE dose), remains unclear.

Indirect host-related cues emitted by both genders of the host can have different spatial-time links with the egg stage. This is particularly important when oviposition occurs far from the adult feeding or mating sites, as is the case with female *N. viridula*, which generally oviposit at sites removed from adult feeding sites (Todd 1989; Colazza et al. 1999b). Under such circumstances, cues associated with host males would be of minimal use to egg parasitoids, because these cues would not help the parasitoids to locate eggs. Instead, egg parasitoid females should optimize their sensitivity to cues produced by host females. The available data support such a scenario, because females of two *Trissolcus* species discriminate between chemical residues left by females or males of their pentatomid hosts (Colazza et al. 1999b; Conti et al. 2004). Similarly, *Telenomus isis* (Polaszek) females are able to discriminate between scales from female or male *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae), showing a strong preference for the former (Chabi-Olaye et al. 2001). However, until now, the nature of the semiochemicals that allow egg parasitoids to distinguish between chemical residues left on substrates by male and female hosts had not been elucidated. The chemical and behavioral evidence provided in this paper suggests that *T. basalis* uses *n*-nonadecane, a sex-specific component of the cuticular hydrocarbons of male *N. viridula*, to discriminate between residues left by males or females. Whereas hexane extracts of the cuticular lipids of adult *N. viridula* showed qualitative and quantitative differences in the composition of the straight-chain hydrocarbon fraction, only nC$_{19}$, the male-specific hydrocarbon, was recovered from extracts of males and from DC-SPME of bug footprints. Furthermore, when nC$_{19}$ was added to the crude extracts of *N. viridula* females, *T. basalis* females showed significantly reduced arena residence times, similar to what occurs when female wasps are presented with hexane extracts of host males.

In summary, we report that hydrocarbons from the host cuticle mediate the responses of an egg parasitoid, and we provide the first identification of a kairomone that acts as a host gender recognition cue. Linear alkanes are common in nature, and they are present in the epicuticular waxes of foliage and other plant tissues, where they play an important role in plant physiology, and in the interaction of plants with other organisms (Müller and Riederer 2005). For example, compounds from a phytophagous insect that are adsorbed on the plant surface are used as host-finding kairomones for insect parasitoids (Müller and Riederer 2005), and leaf epicuticular waxes influence the ability of parasitoids to forage, locate, and attack their hosts (McAuslane et al. 2000; Grant and Barbosa 2006). What remains unknown is the mechanism by which an egg parasitoid can distinguish hydrocarbon residues from its host insects amid the waxy background of the plant cuticle. A better understanding of the key compounds involved in the host location process of insect parasitoids could not only provide fundamental ecological knowledge, but also may allow us to improve insect parasitoid efficiency by manipulation of their behavior with reconstructed blends of the relevant kairomones with synthetic compounds.

Acknowledgments We thank Giampiero La Rocca for providing Trypsin-EDTA. This work was financially supported by Miur Cofin 2004. It is part of the European Science Foundation (ESF) Behavioral Ecology of Insect Parasitoids (BEPAR) scientific program.

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Keywords (3*R*, 7*R*)-3,7-diacetoxy-docosanoic acid · Oncidinol · *Byrsonima intermedia* · Malpighiaceae · *Tetrapedia diversipes* · Apideae · Oncidiinae · Orchidaceae

Introduction

Vogel (1969, 1974) first described floral oils as bee floral rewards. These floral oils are known to have evolved independently in 10 different families of angiosperms: Orchidaceae, Iridaceae, Krameriaceae, Malpighiaceae, Primulaceae, Scrophulariaceae, Solanaceae, Caesalpiniaceae, Gesneriaceae, and Curcubitaceae (Seigler et al. 1978; Steiner 1998).

Particularly interesting are the oil-producing orchids belonging to the subtribe Oncidiinae (Whitten et al. 2000). In the neotropics, many *Oncidium* s.l. (Orchidaceae: Oncidiinae) mimic the flowers of malpighiaceous species. Members of both groups bloom simultaneously and have functionally similar inflorescences, consequently attracting and sharing visitors and pollinators, mainly floral oil-collecting bees (Nierenberg 1972; Williams et al. 2001; Whitten 2006; Silvera 2002). Floral oil-collecting bees, most of which are solitary bees, have evolved independently in five different groups of two bee families: Melittinae (Melittidae), Ctenoplectrini, Centridini, Tapinotaspidini, and Tetrapediini (Apidae) (Steiner 1998; Sigrist and Sazima 2004; Vinson et al. 1997, 2006). These solitary bees presumably apply the oil as it is collected, or with some of their own secretions, to the cell provisions that occupy a portion of the brood cell (Alves dos Santos et al. 2002).

Pollination of many Oncidiinae and malpighiaceous species are mediated by oil-collecting bees. Field observations have revealed pollinaria of an *Oncidium* sp. on the head of *Tetrapedia diversipes* (Alves dos Santos et al. 2002). In addition, *Tetrapedia diversipes* has been observed collecting floral oil from *Oncidium cornigerum* and *O. pubes*, both Oncidiinea species growing at UNICAMP (Singer 2003), and from Malpighiaceae flowers (*Tetrapedia* sp, Neff and Simpson 1981; Sigrist and Sazima 2004).

Previous investigations on the floral oil chemistry of Oncidiinae species revealed that major constituents are asymmetrically substituted diacylglycerols, with a 3, 7- disubstituted fatty acid moiety possessing either acetoxy and/or hydroxyl groups (Reis et al. 2000, 2003, 2006; Reis 2005). However, there is no chemical evidence of *T. diversipes* visiting both Oncidiinae sp and Malpighiacea species and using these oils for survival. Thus, our primary objective was to explore the oil-pollination syndrome by comparing major lipid constituents of a solitary bee nest provision, *T. diversipes* Klug , located close to *Byrsonima intermedia* (Malpighiaceae) and several previously studied Oncidiinae species (*Oncidium pubes*, *Ornithophora radicans*, *Phymatidium delicatulum*, *Phymatidium tillandesioides*, Reis et al. 2000, 2003, 2006).

Methods and Materials

Collection, Extraction, and Purification of *Byrsonima* Floral Oils

Floral oil from *B. intermedia* (Voucher # UEC 95.102) growing at the University of São Paulo (São Paulo, Brazil), December 2003, was collected by washing the flowers with ethyl acetate (10 ml of solvent, 10 flowers, 2 mg of residue after solvent evaporation). Thin layer chromatography revealed one major spot (Rf 0.1, ethyl acetate/hexane 2/8) compatible with the presence of carboxylic acid derivatives. As previously reported for Oncidiinae floral oils (Reis et al. 2000, 2003, 2006; Reis 2005), the unaltered mixture and the mixture methylated with diazomethane were analyzed by ^{1}H NMR. Based on these data, we suggested that the major floral oil constituents were fatty acid derivatives possessing no glycerol moiety.

For a more complete study, fresh flowers (50 g) were dipped into ethyl acetate (40 ml) for 1 min. The ethyl acetate extract (0.68 g) obtained by evaporating the organic solvent was methylated with diazomethane. Purification of the crude extract by silica column chromatography (15 g), eluted with 700 ml of hexane/ethyl acetate (7.5:2.5) furnished a pure methyl ester (35 mg), whose acid moiety is herein named byrsonic acid **1a** (Fig. 3). Colorless oil, $[\alpha]_D^{20} + 4,0(c)0.2$, $CHCl_3$), IR νmax (film) 2923.82, 2852.16, 1740.6, 1458.97, 1430.99, 1241.58, 1022.93 cm-1; HRMS 470.3601 found (calculated 470.3607); 378.343 (16%), 350.313 (36%), 157.078 (100%) , 128.077 (43%). ^{1}H and ^{13}C NMR (Table 1).

Byrsonic Acid Absolute Configuration

Hydrolysis of Byrsonic Acid

Hydrolysis of **1a** (30 mg) in THF/MeOH/H_2O (3:1:1) in the presence of LiOH (Reis et al. 2003) furnished 3,7-dihydroxy-docosanoic acid, which was methylated with diazomethane,

Table 1 1D and 2D NMR [^{1}H (500 MHz, $CDCl_3$) and ^{13}C (125 MHz, $CDCl_3$)] data (HSQC, HMBC) of compound **1a** and 1D NMR ^{1}H (300 MHz, $CDCl_3$) and ^{13}C (75 MHz, $CDCl_3$)] data of **2a** and **3a**

Compound **1a**				Compounds **2a** and **3a**		
C#	δ_C	δ_H (mult, J)	HMBC	C#	δ_C	δ_H (mult, *J*)
1′	51.75	3.68 (s, 3H)		CH_3O	51.82	3.78 (s, 3H)
1	170.36			1	173.23	
2	38.89	2.53 (dd, *J*=5.5 and 15.6 Hz, 1H) 2.58 (dd, *J*=7.6 and 15.6 Hz, 1H)	C1, C3, C4	2	41.13	2.53 (dd, *J*=3.30 and 16.48, 1H) 2.42 (dd, *J*=8.79 and 16.48, 1H)
3	70.18	5.20 (m, 1H)	C1, C5	3	67.86	4.02 (m, 1H)
4	33.70	1.61 (m)	C3, C5, C2	4	36.22	1.47 (m)
5	20.92	1.38 (m, 1H) 1.25 (m, 1H)	C4, C6	5	21.46	1.47 (m)
6	33.75	1.53 (m. 2H)	C7	6	37.52	1.47 (m)
7	73.89	4.84 (m, 1H)	*CO*CH_3-7	7	71.80	3.59 (m)
8	34.04	1.53 (m, 2H)	C7	8	37.09	1.47 (m)
9	25.25	1.25 (m)		9	25.65	1.25 (m)
10	29.32	1.25 (m)		10	29.35	1.25 (m)
11	29.42	1.25 (m)		11–17[a]	29.68	1.25 (m)
12–13	29.64	1.25 (m)		18[a]	31.91	1.25 (m)
14–15	29.62	1.25 (m)		19[a]	22.68	1.25 (m)
16–17	29.61	1.25 (m)		20[a]	14.12	0.87 (t, *J*=7.0 Hz, 3H)
18	29.53	1.25 (m)		*H*OC-3		2.97 (bs, 1H)
19	29.48	1.25 (m)		*H*OC-7		2.04 (bs, 1H)
20	31.87	1.25 (m)				
21	22.64	1.25 (m)				
22	14.07	0.87 (t, *J*=6.6 Hz, 3H)				
COCH_3	170.89					
CO*C*H_3	21.16	2.04 (s, 3H)				
COCH_3	170.76					
CO*C*H_3	21.02	2.03 (s, 3H)				

[a] For **3a** are 11–18, 19, 20, 21, and 22, respectively

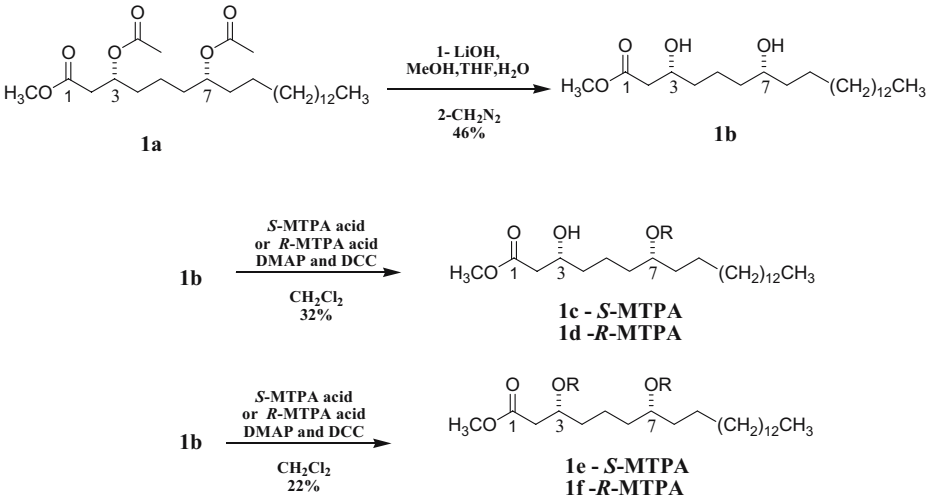


Fig. 1 Scheme for the preparation of **1b**–**1f**

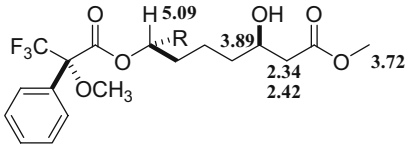
producing methyl 3,7-dihydroxy-docosanoate **1b** (10.6 mg, 46% yield) (Fig. 1). ^{1}H NMR (300 MHz, $CDCl_3$, d_{TMS}) *d*: 4.05 (m, 1H, H-3), 3.71 (s, 3H, CH_3O), 3.60 (m, 1H, H-7), 2.97 (bs, 1H, OH-3), 2.52 (dd, *J*=3.30 and 16.48, 1H, H-2) 2.42 (dd, *J*=8.79 and 16.48, 1H, H-2), 1.40 (m, 6H. H-4, H-6 e H-8) 1.30 (m, 2H, H-5), 1.25 (m, 24H, H-9-H-21), 0.87 (t, *J*=6.23 Hz, 3H, H-22). ^{13}C NMR (75 MHz, $CDCl_3$, d_{TMS}) *d*: 14.26 (C-22), 21.60 (C-5), 22.81 (C-21), 25.79 (C-9), 29.47 (C-10), 29.80 (C-11-C-19), 32.03 (C-20), 37.64 (C-8), 37.21 (C-6), 36.47 (C-4), 41.13 (C-2), 51.82 (CH_3O), 67.91 (C-3), 71.86 (C-7), 173.23 (C-1). MS, (70 eV): *m/z* 350 (3%), 295 (7%), 157 (100%), 128 (69%), 125 (22%), 103 (24%), and 43 (29%).

Preparation of (S)- and (R)-methoxy-(trifluoromethyl)-phenylacetic acid (MTPA) Ester Derivatives of Byrsonic Acid, **1c** *and* **1d**

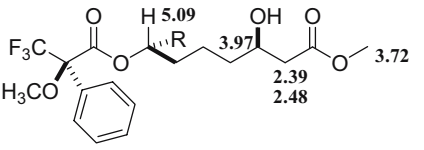
To a solution of **1b** (1 mg, 2.6 mmol) in CH_2Cl_2 (1 ml), *S*-(-)- (MTPA acid) (0.91 mg, 3.9 mmol), 4-dimethylaminopyridine (DMAP) (0.005 mg, 0.003 mmol), and 1,3-dicyclohexylcarbodiimide (DCC) (0.8 mg, 3.9 mmol) were sequentially added at 0°C (Fig. 1). The mixture was stirred for 15 hr, under N_2 at room temperature and then purified on a silica gel column eluted with hexane/ethyl acetate (8:1). The solvent was removed under reduced pressure to give the *S*-Mosher esters **1c** (0.5 mg, 32% yield). Treatment with *R*-(-)-methoxy-(trifluoromethyl)phenylacetic acid as described above yielded the Mosher ester **1d** (0.6 mg, 38% yield). ^{1}H NMR data are in Fig. 2.

Preparation of (S)- and (R)-MTPA Ester Derivatives of Byrsonic Acid, **1e** *and* **1f**

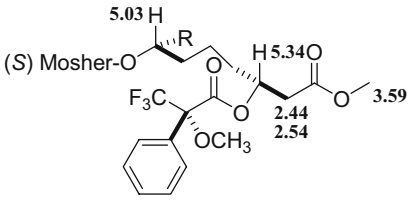
To a solution of **1b** (1.70 mg, 4.40 mmol) in CH_2Cl_2 (1 ml), *S*-(-)-methoxy-(trifluoromethyl) phenylacetic acid (MTPA) (2.6 mg, 11 mmol), 4-dimethylaminopyridine (0.0054 mg, 0.044 mmol), and 1,3-dicyclohexylcarbodiimide (2.27 mg, 11 mmol) were sequentially added at 0°C (Fig. 1). The mixture was refluxed for 24 hr under N_2 and then purified on a silica gel column chromatography eluted with hexane/ethyl acetate (8:1). The solvent was removed



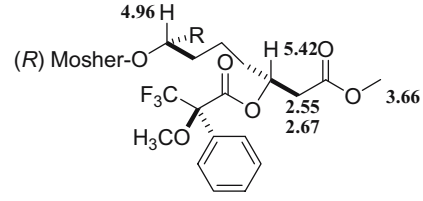
1c R=$C_{15}H_{31}$



1d R=$C_{15}H_{31}$



1e R=$C_{15}H_{31}$



1f R=$C_{15}H_{31}$

Fig. 2 Partial ^{1}H NMR (500 MHz, $CDCl_3$) data of the (*S*)- and (*R*)-Mosher ester derivatives **1c**, **1d**, **1e**, and **1f**. Chemical shifts are referenced to TMS (*d* 0.00)

under reduced pressure, to give the *S*-Mosher esters **1e** (0.8 mg, 22% yield). Treatment with *R*-(-)-methoxy-(trifluoromethyl)phenylacetic acid as described above yielded the Mosher ester **1f** (0.8 mg, 22% yield). ^{1}H NMR are in Fig. 2.

Collection, Extraction, and Purification of *Tetrapedia diversipes* Nests

Trap nests were set at the University of São Paulo in São Paulo, Brazil close to Malpighiaceae and Oncidiinae species. Bamboo pieces (15 cm long, 2 cm diam) closed at one end were used for nest construction. The *T. diversipes* nest was collected as soon as the female closed the cell. The bee cell provisions (79 mg) were extracted with methanol for 48 hr. The crude methanol extract (34 mg) was methylated with diazomethane. This was extracted with ethyl acetate (3 mg) and analyzed by gas chromatography-mass spectroscopy (GC-MS) revealing the presence of two major components, **2a** (20%) and **3a** (33%). The methylated crude provision extract (3 mg) was purified by silica gel column chromatography, yielding a mixture of **2a** and **3a** (1 mg), methyl esters derivatives of **2** and **3**. Compound **2a**: (MS, 70 eV): *m/z* 322 (2%), 267 (7%), 157 (100%), 128 (69%), 125 (45%), 103 (26%), and 43 (21%). Compound **3a**: (MS, 70 eV): *m/z* 350 (3%), 295 (7%), 157 (100%), 128 (69%), 125 (20%), 103 (33%), and 43 (24%). ^{1}H and ^{13}C NMR (Table 1).

Instruments

^{1}H NMR spectra were recorded with a Varian INOVA instrument operating at 500 MHz. Tetramethylsilane (TMS; 0.0 ppm) was used as an internal reference. ^{13}C NMR spectra were obtained at 125 MHz in $CDCl_3$, and the reference signal was from the solvent (77.0). GC-MS analyses were carried out by using an Agilent-6890/5973 system equipped with an HP-5 fused silica capillary column (30 m×0.25 mm×0.25 μm). Oven temperature was programmed from 50 to 290°C at 4°C min^{-1}. Injector temperature was 240°C. Helium was used as carrier gas, flow rate 1 ml min^{-1}. Mass spectra were taken at 70 eV with a scanning

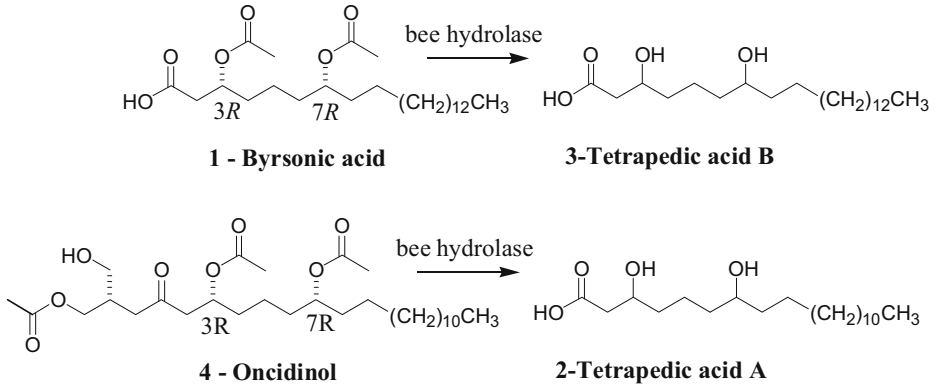


Fig. 3 Byrsonic acid **1** isolated from *Byrsonima intermedia*, Tetrapedic acid A **2** and Tetrapedic acid B **3** isolated from the nest of *Tetrapedia diversipes*, and ondicinol **4** isolated from *Ornithophora radicans* and detected in several other Oncidiinae floral oils (Reis et al. 2003)

speed of 0.84 scans s^{-1} and from *m*/*z* 40 to 700. High-resolution mass spectra at 70 eV were measured on a Micromass VG Autospec mass spectrometer.

Results and Discussion

Byrsonima Intermedia Floral Oil

^{1}H NMR and TLC analyses of *B. intermedia* floral oil revealed that this oil is mainly comprised of free fatty acids. The major fatty acid was isolated by silica gel column chromatography and was named byrsonic acid. Spectral data analysis of byrsonic acid methyl ester **1a** (^{1}H and ^{13}C NMR, 1D and 2D, HSQC, Table 1) revealed two methyl groups belonging to acetoxy groups (d_c 21.20, d_H 2.04; and d_c 21.16, d_H 2.03) and two carbinolic carbons at d_c 70.18 (d_H 5.19, m) and d_c 73.89 (d_H 4.84, m). Analysis of the long-range heteronuclear correlations (H, C HMBC, Table 1) provided evidence that one of the acetoxy groups was located at carbon 3 (d_H 1.61, d_C 38.89). The position of the second acetoxy group was not easily assigned because of a hydrogen signal superposition in the 1D (^{1}H NMR) and 2D NMR (H,H COSY) spectra, mainly between d_H 1.61–1.29. The correlation, J^{1} d_H 1.25 (overlapped) and 1.28, d_c 20.92, in the 2D NMR spectrum (H.C HSQC) indicated the presence of two diastereotopic hydrogens belonging to a methylene carbon (d_c 20.92). The unequivocal proof of this methylene neighborhood was obtained by long-range heteronuclear correlations (H,C HMBC) of hydrogens at d_H 1.38 (weak, ^{3}J, H-5), d_H 1.61 (^{2}J, H-4), d_H 2.53–2.58 (^{2}J, H-2) and carbon at d_c 70.18 (C-3) (Table 1). The weak correlation was assigned to methylene C-5 (d_c 20.92, d_H 1.38 and 1.25). In addition, a ^{2}J coupling between hydrogen at d_H 1.61 (H-4) and carbon at d_c 20.92 (C-5) indicated that this carbon is the C-3–C-5 moiety, and its small chemical shift was assigned to the double shielding *g*-gauche effect of the substituents at C-3 and at C-7 (Duddeck 1986). Unequivocal proof tethering all hydrogens of C-2 to C-7 was achieved by total correlation spectroscopy 1D-TOCSY exciting H-3 (d_H 5.20) by applying shaped pulses and 100 msec of mixing time (optimized value). Therefore, all hydrogen spin systems from C-2 to C-7 were visualized d_H 4.84 (H-7), 2.53 (H-2), 2.58 (H-2), 1.61 (H-4), 1.53 (H-6), 1.38 (H-5), 1.25 (H-5). A second 1D-TOCSY experiment exciting hydrogen-7 (d_H 4.84) and a mixing of 100 msec produced the same ensemble of signals d_H 5.20 (H-3), 2.53 (H-2), 2.58

(H-2), 1.61(H-4), 1.53 (H-6), 1.38 (H-5), 1.25 (H-5, H-8, H-9, H-10). However, the signal amplitude at d_H 1.25 was higher in the second experiment, which is compatible with its neighborhood (aliphatic CH_2 d_H 1.25). Consequently, the suggested structure for byrsonic acid, a novel natural fatty acid, is 3,7-diacetoxy-docosanoic acid **1**.

To determine the absolute configuration of the two asymmetric centers, we applied Mosher's methodology (Dale and Mosher 1973; Sullivan et al. 1973; Ohtani et al. 1991) by strategically modifying one chiral center at a time (Reis et al. 2003) and keeping in mind that the observed Δd^{SR} for certain hydrogens is the sum of the anisotropic effects of all MTPA present, leading to a result that is different from the Δd^{SR} obtained for the polyols with only one MTPA residue. By taking into consideration the above strategy, we first hydrolyzed compound **1a** producing methyl 3,7-dihydroxy-docosanoate **1b**. The selective esterification of hydroxyl-7 of the methyl 3.7-dihydroxy-docosanoate **1b** with (*S*)-MPTA and (*R*)-MPTA produced the derivatives **1c** and **1d**, which were characterized by the H-7 signals at d_H 5.09. The chemical shifts and the difference Δd^{SR} are depicted in Fig. 2. From these results, we suggest a 7-*R* configuration for **1**. To determine the C-3 absolute configuration of **1**, we used the 3,7-di-Mosher ester derivatives **1e** and **1f**. By scrutinizing **1c** and **1d**, we observed that the (*R*)- and (*S*)-MTPA derivatization had little influence on the methoxy group chemical shift of **1c** and **1d**. Therefore, the negative Δd^{SR} values observed in **1e** and **1f** were assigned to the anisotropic influence of the ester group on C-3 (Fig. 2). As a general rule, the effect on C-2 (β-carbonyl) hydrogens of both **1e** and **1f** was 50% larger than in **1c** and **1d**, which allowed a straightforward evaluation of the anisotropic effect arising from the Mosher acid group on C-3. From these observations, we suggest a 3-*R* absolute configuration for compound **1**. Consequently, the novel fatty acid from *B. intermedia*, herein named byrsonic acid, is (3*R*, 7*R*)-3,7-diacetoxy-docosanoic acid **1** (Fig. 3).

Tetrapedia diversipes Nest

Structures **2** and **3** were suggested for the two major components present in the *T. diversipes* nest cell provisions based on spectroscopic characteristics of their methyl ester derivatives **2a** and **3a** (^1H and ^{13}C NMR—Table 1 and MS). The two carbinols at d_c 67.86 (d_H 4.02, m) and d_c 71.81 (d_H 3.59, m) and the methylene carbon (d_c 21.46) were assigned to C-3, C-7, and C-5 of **2a** and **3a**. These spectral features were similar to those displayed by the methylated fatty acid moiety of **1b**. Based on the NMR and MS data, the structures were inferred to be 3,7-dihydroxy-eicosanoic acid (**2**) and 3,7-dihydroxy-docosanoic acid (**3**), two new fatty acids derivatives named tetrapedic acid A and B, respectively.

Byrsonima intermedia (Malpighiaceae), *Ornithophora radicans* (Oncidiinae, Orchidaceae) and *Tetrapedia diversipes* (Tetrapediini, Apidae) Chemical Similarities

Byrsonic acid (**1**) was identified in *B. intermedia* and Oncidinol (**4**) in several Oncidiineae species (*Oncidium hookeri*, *O. longicornu*, *O. varicosum*, *Phymatidium tillandensioides*, and *P. delicatum*) (Reis et al. 2000, 2003, 2006; Reis 2005). These findings are remarkable because both tetrapedic acid A **2**, and tetrapedic acid B **3** can be derived from Oncidiinae and Malpighiaceae species floral oils, which are biotransformed by hydrolases secreted by female mandibules during nest food mass mixing and manipulating (Fig. 3). It has been reported (Alves-Dos-Santos et al. 2002) that females spend a few minutes manipulating and consolidating the collected floral material. Thus, nest building involves not only floral oil harvesting, but also mixing it with secretions that could perform the chemical modifications we have observed (Alves-Dos-Santos et al. 2002). It is not yet clear why plants of unrelated

families such as Orchidaceae and Malpighiaceae produce these rare fatty acids: is it mimicry that lures the bees adapted to Malpighiaceae flowers to visit Orchidaceae?

In summary, this research has revealed that the main constituent of *B. intermedia* floral oil is (3*R*, 7*R*)-3,7-diacetoxy-docosanoic acid (**1**). The similarity of this compound with Oncidinol (**4**), present in the floral oil of Oncidiinae species is intriguing because both possess the same 3,7 substitution pattern and the same 3*R* and 7*R* absolute configurations. The same substitution pattern is present in major fatty acids isolated from *T. diversipes* cell provisions, 3,7-dihydroxy-eicosanoic acid (**2**) and 3,7-dihydroxy-docosanoic acid (**3**), presently named tetrapedic acids A and B, respectively. Both tetrapedic A (C20) and B (C22) may be biotransformation products of Oncidinol and Byrsonic acid by *T. diversipes* hydrolases in the hydropharingeal glands. These are clues to bee visitation and oil collecting from both Oncidiinae and Malpighiacea species, thus indicating that the deceit/pollination syndrome is not true for all Oncidiinae flowers. This is new information on the Apidae/Oncidiinae/Malpighiaceae relationships from a chemical point of view, a subject that has been attracting the attention of biologists since 1969 (Vogel 1974).

Acknowledgements The authors are indebted to FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo—grants #01/02794-7, #98/07781-6 and #03/06624-4) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnologia) for scholarships and financial support. The authors are also grateful to Vera Lúcia Imperatriz Fonseca and Sandra Regina Capelari Naxara of Universidade de São Paulo-USP for the *Byrsonima intermedia* flowers, and Carol Collins and Volker Bittrich for critical reading and text correction.

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monolignol, lignans, phenylethanoids, and secoiridoids. Both qualitative and quantitative differences were observed among the three ash species. Hydroxycoumarins and the phenylethanoids, calceloariosides A and B, were present only in the phloem of Manchurian ash and might represent a mechanism of resistance against EAB.

Keywords *Agrilus planipennis* · LC-MS · Hydroxycoumarins · Phenolics · Coevolution · Host resistance · Invasive species

Introduction

Since its accidental importation from Asia, emerald ash borer (EAB *Agrilus planipennis* Fairmaire (Col: Buprestidae), a phloem-feeding beetle, has infested and killed millions of ash (*Fraxinus* spp.) trees in Indiana, Ohio, Michigan, and Ontario (Herms et al. 2004; Cappaert et al. 2005; Poland and McCullough 2006). As EAB continues to spread, it has the potential to eliminate ash species from natural and urban forests throughout North America. In Ohio alone, there are 3.8 billion white ash trees (Griffith et al. 1991). Unlike congeneric wood-boring beetles endemic to North America, including bronze birch borer (*Agrilus anxius* Gory) and two-lined chestnut borer (*Agrilus bilineatus* Weber) that colonize stressed trees (e.g., Anderson 1944; Dunn et al. 1990), EAB is more aggressive, killing even healthy trees on high quality sites (Haack et al. 2002; Herms et al. 2004; Poland and McCullough 2006).

In a recent common garden study that investigated interspecific variation in ash resistance to EAB, Manchurian ash (*Fraxinus mandshurica* Rupr.) was shown to have low levels of EAB colonization and EAB-induced mortality, while North American ashes experienced high rates of colonization and severe mortality (Rebek et al. 2006). These observations are consistent with the hypothesis that Asian ashes have high resistance to EAB by virtue of their coevolutionary history. Reports indicate that EAB is rare in Asia, where Manchurian ash is a primary host (Haack et al. 2002; Liu et al. 2003; Schaefer 2005), and infestations appear restricted to stressed trees (Gould et al. 2005). This implies that Asian ashes may be generally resistant.

Relatively little research has addressed mechanisms of resistance of angiosperm trees to wood-borers (e.g., Hanks et al. 1999). We propose that in the case of phloem-feeding buprestids, host resistance results from constitutive and induced physical and phytochemical defenses (Dunn et al. 1990). More specifically, defense may have three main components: (1) constitutive, or pre-attack, physical and chemical defenses, such as thick outer bark and preexisting pools of compounds in the phloem that are either toxic and/or serve as deterrents against the insect; (2) immediate, attack-induced *de novo* synthesis and accumulation of specific, low-molecular-weight secondary metabolites, phenolic polymers such as lignin and suberin, and defensive proteins (Kemp and Burden 1986; Bostock and Stermer 1989; Fernandes 1990; Pearce 1996; Kehr 2006), which may slow the growth of the invading organism enough to allow for (3) subsequent formation of wound (necrophylactic) periderms (i.e., callus tissue), which serve to isolate the wound, inhibit the spread of the colonizing organism (e.g., by encapsulation of insect larvae), and reestablish phellogen and cambial integrity (Mullick and Jensen 1973; Mullick 1977; Biggs et al. 1984; Bostock and Stermer 1989; Robinson et al. 2004).

Allelochemicals are represented by a diverse array of plant secondary metabolites, including terpenes and phenolic derivatives such as tannins, flavonoids, and stilbenes. Whether constitutively expressed or induced in response to herbivory, they may serve a wide range of roles. The genus *Fraxinus* belongs to the family Oleaceae, which comprises around 600

species in 25 genera (Wallander and Albert 2000; Perez et al. 2005). The olive (*Olea europaea* L.), cultivated for its fruit and oil, belongs to this family and has been studied extensively with regard to chemical constituents. Compound classes that have been reported to be commonly associated with olives include iridoid glucosides, phenylethanoids, coumarins, and lignan glucosides (Bianco et al. 2001; Jensen et al. 2002; Ryan et al. 2002; Cardoso et al. 2005).

As part of a broader study investigating potential mechanisms of Manchurian ash resistance to EAB, we compared the constitutive composition of secondary metabolites in phloem from the main stem of white (*Fraxinus americana* L.), green (*Fraxinus pennsylvanica* Marsh.), and Manchurian ash. We hypothesized that: (a) Manchurian ash phloem contains concentrations of particular constitutive secondary metabolites different from white and green ash, and (b) that these compounds may be involved in resistance. To date, relatively few studies have investigated the phenolic chemistry of *Fraxinus* spp. phloem (Terazawa and Sasaya 1970; Tsukamoto et al. 1984; 1985; Nykolov et al. 1993; Kostova et al. 1995; Iossifova et al. 1998, 1999) with many focused on manna ash (*Fraxinus ornus* L.) (Steinegger and Brantschen 1959; Kostova 2001). The present paper addresses hypothesis (a), by providing chemical data that will be needed to investigate the role of putative defense compounds in resistance of Manchurian ash to EAB (hypothesis b).

Methods and Materials

Plant Material Eight 4-yr-old saplings each of *F. americana*, *F. mandshurica*, and *F. pennsylvannica* were obtained from Bailey Nursery, Inc., St Paul, MN, USA. The ash saplings had a mean stem height of 181±0.03 (SEM), 216±0.06, and 149±0.03 cm, respectively, and a mean stem diameter of 15.8±0.5, 23.2±0.9, and 12.4±0.3 cm, respectively. The dormant bare-root saplings were stored in the dark at 4°C for 2 wk before sampling.

Chemicals and Reagents Methanol and acetic acid (both HPLC grade) were purchased from Fisher (Pittsburg, PA, USA). Ultrapure water was prepared using a Milli-Q water system (Millipore Ltd, Bedford, MA, USA). Standards of caffeic acid, esculin, and vanillin were purchased from Sigma Aldrich (St. Louis, MO, USA). Chlorogenic acid, fraxin, oleuropein, and verbascoside, were purchased from Extrasynthese (Genay, France). Pinoresinol was purchased from Arbo Nova (Turku, Finland).

Extraction One hundred milligrams of stem phloem were ground in liquid nitrogen and extracted twice with 500 μl of 100% methanol over 48 hr in the dark at 4°C. The pooled extract was transferred to a 1.5-ml microcentrifuge tube and centrifuged (12,000×*g* for 5 min) to remove solids. Samples were stored at −20°C and analyzed within 1 wk of extraction.

Analysis of Phenolics with HPLC-UV, HPLC-ESI-MS, and ^{1}H NMR and ^{13}C NMR HPLC with UV detection and HPLC-ESI-MS were used to analyze phenolics. LC-MS analyses were conducted on a Hewlett Packard HPLC system (Palo Alto, CA, USA) model HP 1100 equipped with an HP DAD G1315A detector coupled to a QTof-1 mass spectrometer (Micromass, Manchester, UK). Chromatographic separation was carried out by using a Waters Xterra™ RP18, 5 μm, 4.6×150 mm column and a Waters Xterra ™ RP18, 3.9 μm, 3.0×20 mm guard column. The autosampler and column temperatures were set at 4 and 30°C, respectively.

The binary mobile phase consisted of water/acetic acid (A) (99.9:0.1, *v*/*v*) and methanol/acetic acid (B) (99.9:0.1, *v*/*v*), and the flow rate was set at 1 ml/min. The elution program was as follows (percentages refer to proportion of eluant B): 5–15% (0–15 min); 15–30% (15–35 min); 30–40% (35–40 min); 40–60% (40–50 min); 60–90% (50–55); 100% (55–60 min). The injection volume for all samples was 5 μl. Compound separation was followed by post-column splitting (1:1), passing of the LC effluent through the Photodiode Array (PDA) Detector (scanning range, 200–400 nm) and an electrospray source (source block temperature, 120°C; desolvation temperature, 400°C). The MS detector was optimized to obtain maximum yields of $[M\text{-}H]^-$ ions of epicatechin/flavonoid standards. The optimized parameters in the negative ionization mode were as follows: capillary voltage, 3.2 kV; cone voltage, 32 V. Argon was used as the collision gas at 20 psi and nitrogen as the nebulizing gas at 15 l/hr. For full-scan MS analysis, the spectra were recorded in the range of *m/z* 100–900.

We applied MS detection in survey scan mode to identify the major compounds in the HPLC eluate, and we compared these peaks with the PDA trace. In this MS method, full MS scans (survey) were analyzed on-the-fly by the instrument to identify the most abundant parent ions. The mode temporarily switched to MS/MS mode to perform daughter scans of the presumptive parent ions. Data acquisition and processing were performed by using a MassLynx version 3.5 data system (Micromass).

The identifications of esculin, fraxin, verbascoside, oleuropein, and apigenin were unequivocally confirmed by spiking samples with the respective standards and comparing retention times and ultraviolet (UV) spectra. Additional mass data for syringin was obtained with a LCQ Classic (Finnigan) by using a slightly modified version of the above LC-MS method. Calceolariosides A and B, oleuropein, and pinoresinol glucoside were isolated by semipreparative HPLC and further purified by normal-phase preparative TLC (1,000 μm thickness silica gel plates) with chloroform:methanol:toluene:acetic acid (80:30:8:2). These compounds were analyzed by ^{1}H NMR and ^{13}C NMR. Spectra were obtained in methanol-d_4 by using a Bruker Avance DRX-400 (400 MHz) spectrometer. ^{1}H NMR and ^{13}C NMR spectra were calibrated to the residual solvent peaks at δH 3.31 and δC 49.0, respectively.

Quantification of Selected Phenolics After identification, the compounds (Table 4) were quantified against external standards as mg g^{-1} FW. Lack of suitable external standards made it impossible to quantify all compounds. Contents were expressed as verbascoside equivalents for calceolariosides A and B, fraxin equivalents for fraxetin and mandshurin, esculin equivalents for esculetin and methylesculin, oleuropein equivalents for ligustroside and oleuropein hexoside, pinoresinol equivalents for pinoresinol dihexoside, pinoresinol glucoside, and syringaresinol. Quantitative HPLC analyses were performed on an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with an autosampler and a 996 Photodiode Array Detector (Waters). The same column and gradient program as described for the LC-MS system were used. The mobile phase consisted of water/acetic acid (A) (98:2, *v*/*v*) and methanol/acetic acid (B) (98:2, *v*/*v*). Monitoring and processing of data were performed at 280 nm.

Statistical Analysis The contents of selected phenolic compounds that were present in two or more ash species were examined by univariate analysis of variance (ANOVA) using SPSS 14.0 for Windows (SPSS Inc., Chicago). Levene's test was used to examine variance equality. Data that did not have equal variances were log-transformed to satisfy this assumption.

Results

Characterization of Phenolic Compounds Among the three ash species, a total of 38 individual compounds were analyzed in detail. The characterization of unknown phenolic compounds was based on comparison of UV, mass spectrometry (MS), MS fragmentation behavior, and published NMR data. Liquid chromatography/tandem mass spectrometry (LC-TMS) with electrospray ionization has proven to be a powerful tool for the characterization of nonvolatile, polar, and thermally labile phenolic compounds obtained from plant extracts (Eyles et al. 2003a, b; Cardoso et al. 2005; March et al. 2006).

Comparison of the ESI-MS/MS data with published data and standards revealed a diverse range of phenolic compounds in the crude phloem extracts including hydroxycoumarins, lignans, phenylethanoids, and secoiridoids. Tables 1, 2, 3 summarize the tentative identification of the chromatographic peaks on the basis of retention times, molecular weights, and the main ions obtained by TMS for compounds in each species. Given that the majority of the compounds detected are known and have been characterized in previous studies (Tables 1, 2 and 3), only brief explanations of the structural analyses of the major compounds are presented (Fig. 1).

Hydroxycoumarins Seven compounds were identified as hydroxycoumarins (**5**, **7**, **8**, **12**, **14**, **15**, and **16**). Most were identified as a glycoside containing one sugar moiety, usually a hexoside as characterized by a neutral loss of 162 Da (Fig. 2). Esculin (**5**) and fraxin (**14**) were identified by comparing with standards. Compound **8** exhibited a $[M\text{-}H]^-$ ion at *m/z* 353 and UV spectrum maxima at 290 and 335 nm. Its MS/MS spectrum included ions at *m/z* 191, which corresponds to a loss of hexose (162 Da) and at *m/z* 176, which corresponds to a loss of a methoxy group (15 Da) from *m/z* 191. These findings are consistent with those reported for methylesculin (Steinegger and Brantschen 1959; Vereecke et al. 1997). Compounds **12** and **15** were tentatively identified as fraxidin hexoside and mandshurin, respectively (Table 2, Fig. 3). Despite both compounds exhibiting the same $[M\text{-}H]^-$ ion at *m/z* 383 and MS/MS spectrum (*m/z* 221, 206), it was possible to identify each compound by comparing their UV spectra to those previously reported (Terazawa and Sasaya 1970; Jensen and Nielsen 1976).

Lignans Compounds **19**, **20**, **26**, and **28** showed maxima of absorption bands at around 281 nm, characteristic of furofuran lignans (Tsukamoto et al. 1984) and similar to the pinoresinol standard. Compounds **19** and **26** were identified as pinoresinol dihexoside and pinoresinol glucoside, respectively. Pinoresinol dihexoside (**19**) displayed a $[M\text{-}H]^-$ ion at *m/z* 681, and its MS/MS spectrum gave ions at *m/z* 519 and 357, originating from successive losses of 162 Da, suggesting the presence of two hexosyl residues. By contrast, pinoresinol glucoside (**26**) yielded only one neutral loss of 162 Da. Furthermore, the ^1H NMR and ^{13}C NMR data of this compound were consistent with the literature values (Bodesheim and Holzl 1997). Compound **22** exhibited a $[M\text{-}H]^-$ ion at *m/z* 523, corresponding to a neutral loss of a hexose moiety (162 Da) and the MS/MS spectrum (*m/z* 329, 359) was consistent with those reported for lariciresinol (Smeds et al. 2006). Thus, this compound was tentatively characterized as lariciresinol hexoside.

Monolignol Compound **10** exhibited an $[M\text{-}H]^-$ ion at *m/z* 371 and its MS/MS gave a $[M\text{-}H\text{-}162]^-$ ion at *m/z* 209, corresponding to the loss of a hexose. Furthermore, the product ion spectrum of *m/z* 371 yielded fragments at *m/z* 194 and 179, indicating the successive losses of 15 Da and the presence of two methoxyl groups (loss of methyl radicals) (Ye et al. 2005).

Table 1 Characterization of phenolic compounds from the phloem of green ash, *Fraxinus pennsylvanica*

No.	Rt (min)	$[M-H]^-$	Fragments m/z (Ordered by Decreasing Intensity)	UV Maxima	Tentative Identification[a]	References
1	5.78	315	153,135, 119, 123	278	Hydroxytyrosol hexoside	Cardoso et al. 2005
3	7.88	299	119, 113, 137, 131, 149, 179, 161	276	Tyrosol hexoside	Kammerer et al. 2005
4	11.10	431	149, 299, 179, 191, 161, 131, 251, 119	276	Unknown	
6	12.67	601	403, 223, 179, 161	sh 240	Elenolic acid derivative	
10	13.96	371	209, 194, 179, 151	265	Syringin	Wang and Sun 2005
13	16.60	377	197, 153	260	Oleuropein aglycone	Cardoso et al. 2005
18	22.17	353	191	325	Caffeoyl-quinic acid	Cardoso et al. 2005
19	27.42	681	519, 357, 161	274	Pinoresinol dihexoside	Boyer et al. 2005
20	29.16	535	373, 211, 343, 181, 193, 179, 151	278	Hydroxypinoresinol hexoside	Iossifova et al. 1998
21	31.50	523	361	278	Unknown	
24	33.10	391	179, 161, 135, 311	sh 290, 331	Unknown	
26	35.39	519	357, 151	278	Pinoresinol glucoside[b]	Ye et al. 2005
28	37.16	417	181, 402, 387, 166	277	Syringaresinol	Evtuguin and Amado 2003
29	37.94	701	539, 377, 275, 307, 437	sh 280	Oleuropein hexoside	Bianco et al. 2001
31	39.64	685	523, 453, 421, 299, 223, 179	330	Nuzhenide	Ryan et al. 2002; Mulinacci et al. 2005
32	40.06	623	461, 161	sh 285, 331	Verbascoside[c]	Cardoso et al. 2005
33	41.37	539	377, 275, 307, 223, 345, 149, 179	sh 281	Oleuropein[b,c]	Tanahashi et al. 1998; Bianco et al. 2001
36	44.62	523	291, 361, 259, 223, 179	sh 281	Ligustroside	Ryan et al. 2002
37	46.37	609	301, 271	255, 353	Quercetin diglycoside	Cardoso et al. 2005
38	54.44	269	149, 151, 225	267, 339	Apigenin[c]	Fabre et al. 2001

sh Shoulder

[a] Tentative identification based on MS data and UV spectrum consistent with literature

[b] Identification confirmed by NMR

[c] Identification based on retention time, mass spectral and UV spectrum consistent with those of standard

Based on these observations and a characteristic UV spectrum with a peak of 265 nm, **10** was tentatively identified as syringin (Wang and Sun 2005).

Secoiridoids Oleuropein (**33**) was identified by comparing with the standard. The ^{1}H NMR and ^{13}C NMR data for this compound were consistent with those found in the literature

Table 2 Characterization of phenolic compounds from the phloem of Manchurian ash, *Fraxinus Mandshurica*

No.	Rt (min)	$[M-H]^-$	Fragments m/z (Ordered by Decreasing Intensity)	UV Maxima	Tentative Identification[a]	References
1	5.69	315	135, 119, 153, 113, 123	280	Hydroxytyrosol hexoside	Cardoso et al. 2005
2	7.03	167	152, 122, 121	276	Homovanillic alcohol	
3	7.77	299	119, 113, 137, 131, 149, 179, 161	276	Tyrosol hexoside	Kammerer et al. 2005
5	11.82	339	177	290, 336	Esculin[b]	Parejo et al. 2004
7	12.74	177	133, 149, 105	sh 290, 335	Esculetin	Concannon et al. 2000
8	13.13	353	191, 176	sh 290, 335	Methylesculin	Vereecke et al. 1997
9	13.36	389	165, 183, 121, 209, 345, 119	Not detected	Oleoside	Cardoso et al. 2005
11	13.89	353	191, 179, 135, 173	287, 335	Caffeoyl-quinic acid	Cardoso et al. 2005
12	15.57	383	221, 191, 163, 135, 206	292, sh 340	Fraxidin hexoside	Jensen and Nielsen 1976
14	17.01	369	207, 191, 354	sh 300, 341	Fraxin[b]	Godecke et al. 2005
15	17.37	383	221, 206, 191, 161	329	Mandshurin	Terazawa and Sasaya 1970
16	20.49	207	193, 165, 109	335	Fraxetin	Liu et al. 2005
19	26.89	681	519, 357, 161	276	Pinoresinol dihexoside	Boyer et al. 2005
21	30.78	523	361	278	Unknown	
22	32.26	521	329, 359	278	Lariciresinol hexoside	Smeds et al. 2006
25	33.18	335	179, 135, 161	276	Unknown	
26	35.01	519	357, 151, 342, 136	278	Pinoresinol glucoside [b]	Ye et al. 2005
27	36.77	477	161, 179, 135, 315	290, 328	Calceolarioside A[c]	Nicoletti et al. 1986
29	37.10	701	539, 377, 275, 307, 437	sh 278	Oleuropein hexoside	Bianco et al. 2001
31	39.11	685	523, 453, 421, 299, 223, 179	not detected	Nuzhenide	Ryan et al. 2002; Mulinacci et al. 2005
32	39.38	623	461, 161	sh 290, 331	Verbascoside[b]	Cardoso et al. 2005
33	41.14	539	377, 275, 307, 223, 345, 149, 179	sh 281	Oleuropein[b,c]	Tanahashi et al. 1998; Bianco et al. 2001
35	42.07	477	161, 315, 135, 179, 251, 281, 221	285, 328	Calceolarioside B[c]	Nicoletti et al. 1986; Ersöz et al. 2002
36	44.50	523	361, 291, 259, 223, 127	sh 280	Ligustroside	Ryan et al. 2002
37	46.01	609	301, 271	256, 353	Quercetin diglycoside	Cardoso et al. 2005

sh Shoulder

[a] Tentative identification based on MS data and UV spectrum consistent with literature

[b] Identification based on retention time, mass spectral and UV spectrum consistent with those of standard

[c] Identification confirmed by NMR

Table 3 Characterization of phenolic compounds from the phloem of white ash, *Fraxinus americana*

No.	Rt (min)	$[M-H]^-$	Fragments m/z (Ordered by Decreasing intensity)	UV Maxima	Tentative Identification[a]	References
1	5.82	315	153, 123, 135	278	Hydroxytyrosol hexoside	Cardoso et al. 2005
3	7.77	299	119, 113, 137, 131, 149, 179, 161	276	Tyrosol hexoside	Kammerer et al. 2005
6	10.98	431	149, 299, 179, 191, 221, 251	275	Unknown	
8	12.27	565	403, 179, 223, 161, 119, 143	239	Elenolic acid derivative	
9	13.36	389	165, 183, 121, 209, 345, 119	266	Oleoside[b]	Cardoso et al. 2005
10	13.36	371	209, 194, 179, 151	265	Syringin[b]	Wang and Sun 2005
13	16.47	377	197, 153	262	Oleuropein aglycone	Cardoso et al. 2005
17	21.60	537	375, 327, 195, 179	270, sh 305	Unknown	
19	27.02	681	519, 357, 161	276	Pinoresinol dihexoside	Boyer et al. 2005
20	28.70	535	373, 343, 211, 181, 193	278	Hydroxypinoresinol hexoside	Iossifova et al. 1998
21	30.96	523	361	278	Unknown	
22	32.10	521	329, 341, 359	278	Lariciresinol hexoside	Smeds et al. 2006
23	32.58	555	273, 393, 307, 361, 179, 181, 375, 419	sh 290, 332	Unknown	
26	35.00	519	357, 151, 136, 342	278	Pinoresinol glucoside[c]	Ye et al. 2005
28	37.16	417	181, 402, 387, 166	277	Syringaresinol	Evtuguin and Amado 2003
29	37.18	701	539, 377, 275, 307, 437	sh 278	Oleuropein hexoside	Bianco et al. 2001
30	38.40	621	179, 161, 459, 487	286, 331	Unknown	
32	39.11	623	461, 161	sh 285, 331	Verbascoside[d]	Cardoso et al. 2005
33	40.98	539	377, 275, 307, 223, 345, 149, 179	sh 281	Oleuropein[c,d]	Tanahashi et al. 1998; Bianco et al. 2001
34	42.00	685	361, 291, 259, 179	277	Ligustroside derivative	
36	44.37	523	291, 361, 223, 179, 127	280	Ligustroside	Ryan et al. 2002
37	45.98	609	301, 271	255, 353	Quercetin diglycoside	Cardoso et al. 2005
38	54.45	269	149, 151, 225, 201	267, 337	Apigenin[d]	Fabre et al. 2001

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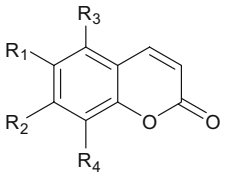
[a] Tentative identification based on MS data and UV spectrum consistent with literature

[b] Peaks co-eluted

[c] Identification confirmed by NMR

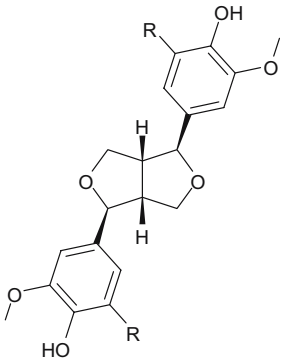
[d] Identification based on retention time, mass spectral and UV spectrum consistent with those of standard

Hydroxycoumarins:



	R_1	R_2	R_3	R_4
Esculin:	Glc	OH	H	H
Fraxin:	OMe	OH	H	Glc
Fraxidin:	OMe	OMe	H	OH
Mandshurin:	Glc	OMe	OMe	H

Lignans:



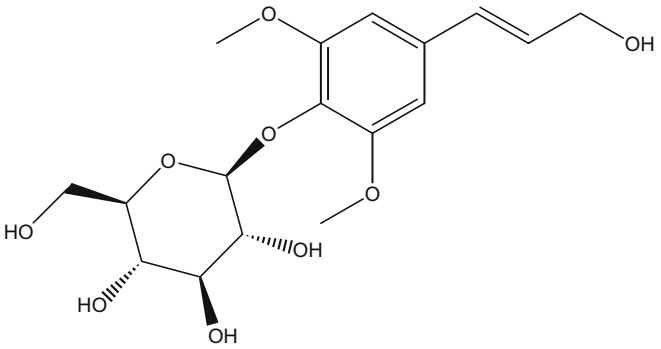
Pinoresinol: R = H
Syringaresinol: R = OMe

Fig. 1 Chemical structure of major compounds in crude ash extracts

(Tanahashi et al. 1998). Compound **29** exhibited the same characteristic UV spectrum as oleuropein but gave an $[M\text{-}H]^-$ ion at *m/z* 701. Its MS/MS spectrum yielded an intense ion at 539, consistent with a neutral loss of a hexose (162 Da) plus other ions (*m/z* 377, 275, 307) identical to those for oleuropein. Therefore, this compound was tentatively identified as oleuropein hexoside. The MS/MS spectrum of compound **36** yielded ions at *m/z* 291, 361, 223, 179, and 127. These findings are consistent with ligustroside (Ryan et al. 2002). Compound **34** exhibited a similar MS/MS spectrum as ligustroside (*m/z* 361, 291, 259, 159) but with a $[M\text{-}H]^-$ ion at *m/z* 685, which corresponds to an addition of 162 Da. Therefore, it was tentatively identified as a ligustroside derivative.

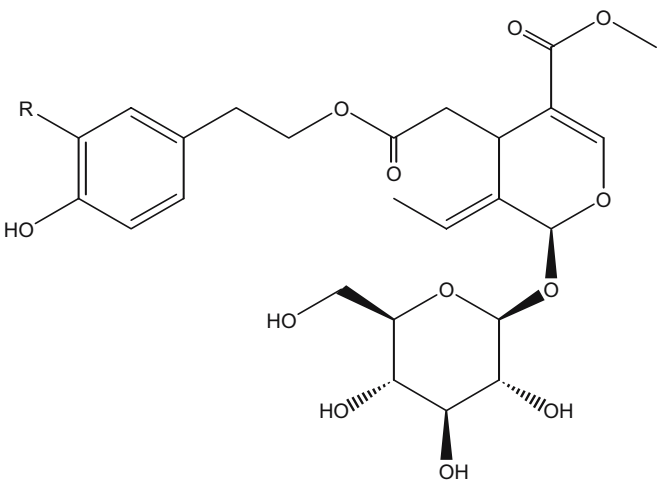
Phenylethanoids Compound **32** was characterized by the $[M\text{-}H]^-$ ion at *m/z* 623 and CID gave two major ions at *m/z* 461 and 161 that are in accordance with the fragmentation of

Monolignol:



Syringin

Secoiridoid glycosides:

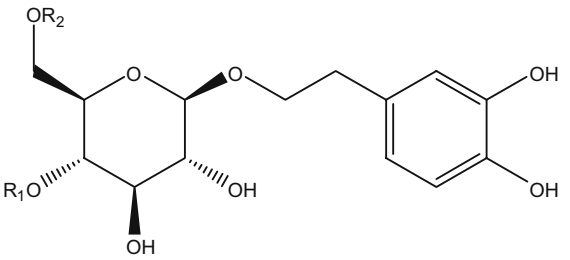


Ligustroside: R = H
Oleuropein: R = OH

Fig. 1 (continued)

verbascoside (Cardoso et al. 2005). Compounds **27** and **35** exhibited $[M\text{-}H]^-$ ion at *m/z* 477 with almost identical UV and mass spectra suggesting them to be isomers of the same compound (Fig. 4). In both cases, their MS/MS spectra gave an $[M\text{-}H\text{-}162]^-$ ion at *m/z* 315,

Phenylethanoids:



Calceolarioside A: R_1 = Caffeoyl R_2 = H
Calceolarioside B: R_1 = H R_2 = Caffeoyl

Fig. 1 (continued)

indicating the loss of a caffeic acid moiety. In addition, caffeic acid (*m/z* 179) and its decarboxylated product (*m/z* 135) generated signals of low intensity (Fig. 4). The intense fragment ion at *m/z* 161 is most likely due to the fragmentation of the hexose moiety by the loss of water (Tolonen et al. 2004). Thus, compounds **27** and **35** were assigned as calceolariosides. The ^{1}H NMR and ^{13}C NMR data showed them to be identical with those reported for calceolariosides A (Nicoletti et al. 1986) and B (Ersöz et al. 2002), respectively.

Comparison of the Three Ash Species Both qualitative and quantitative differences were observed among the three species (Fig. 2, Tables 1, 2, 3 and 4). Comparisons of 17 individual phenolic compounds revealed a number of differences in composition as listed in Table 4. In particular, Manchurian ash was distinguished from the North American ash species by the presence of hydroxycoumarins, particularly mandshurin (**15**), fraxin (**14**), and fraxidin hexoside (**12**), in decreasing order of abundance. Other minor hydroxycoumarins that were not detected in either green or white ash included esculin (**5**), esculetin (**7**), methylesculin (**8**), and fraxetin (**16**). The phenylethanoids, calceolariosides A (**27**) and B (**35**), also were only found in Manchurian ash.

The main lignan in green and white ash was syringaresinol (**28**) followed by smaller amounts of pinoresinol glucoside (**26**) and pinoresinol dihexoside (**19**). Manchurian ash contained amounts of pinoresinol dihexoside (**19**) that were 11-fold higher than in green and 10-fold higher than in white ash ($F_{2,23}$=238, P<0.001). Similarly, higher amounts of pinoresinol glucoside (**26**) were found in Manchurian ash than either green (threefold) or white ash (twofold) ($F_{2,23}$=30.3, P<0.001). By contrast, both green and white ash contained over six times more oleuropein hexoside (**29**) than Manchurian ash ($F_{2,23}$=254, P<0.001). No syringaresinol (**28**) was detected in Manchurian ash. White ash had a higher amount of verbascoside (**32**) than either green (twofold) or Manchurian (ninefold) ash ($F_{2,23}$=28.7, P<0.004). From comparison of the chromatographic profiles, both white and

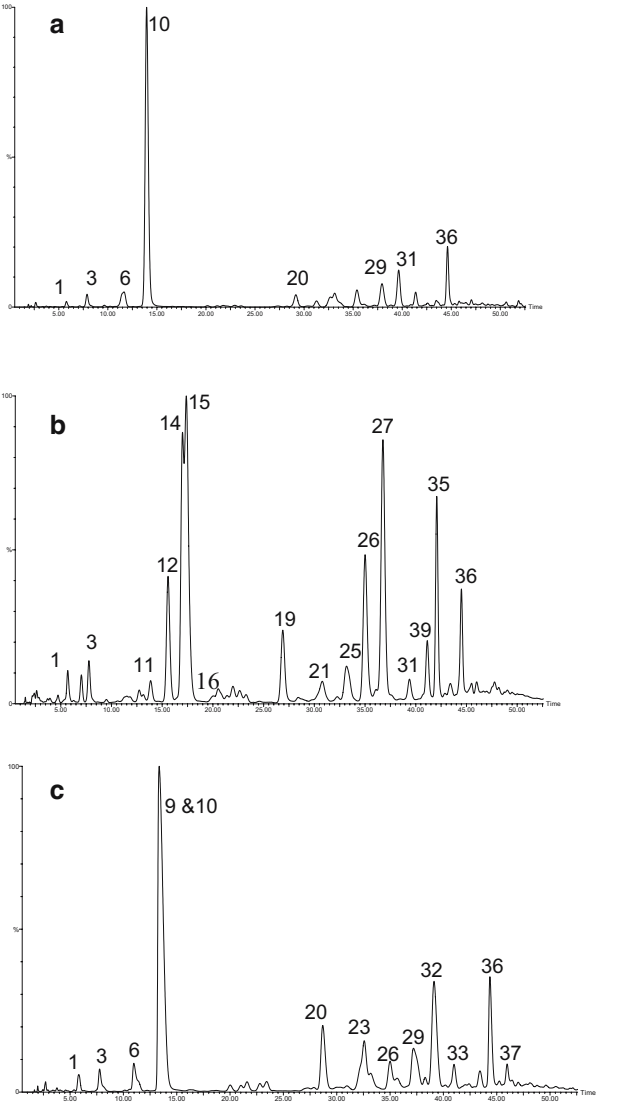


Fig. 2 Comparison of HPLC-UV chromatographic profiles at 280 nm of constitutive phloem extracts (100% methanol) of **a** green ash *Fraxinus pennsylvanica*, **b** Manchurian ash *Fraxinus mandshurica*, and **c** white ash *Fraxinus americana*. For peak assignments of the major peaks, see Tables 1, 2 and 3

green ash appeared to be dominated by the monolignol, syringin (**10**) (Fig. 2). This compound was not present in Manchurian ash.

Discussion

Manchurian ash was distinct from the two Northern American ash species by its unique content of hydroxycoumarins (particularly fraxin, mandshurin, and fraxidin hexoside), calceoloariosides A and B, and significantly higher concentrations of pinoresinol glucoside and pinoresinol dihexoside. The lack of hydroxycoumarins in green and white ash phloem

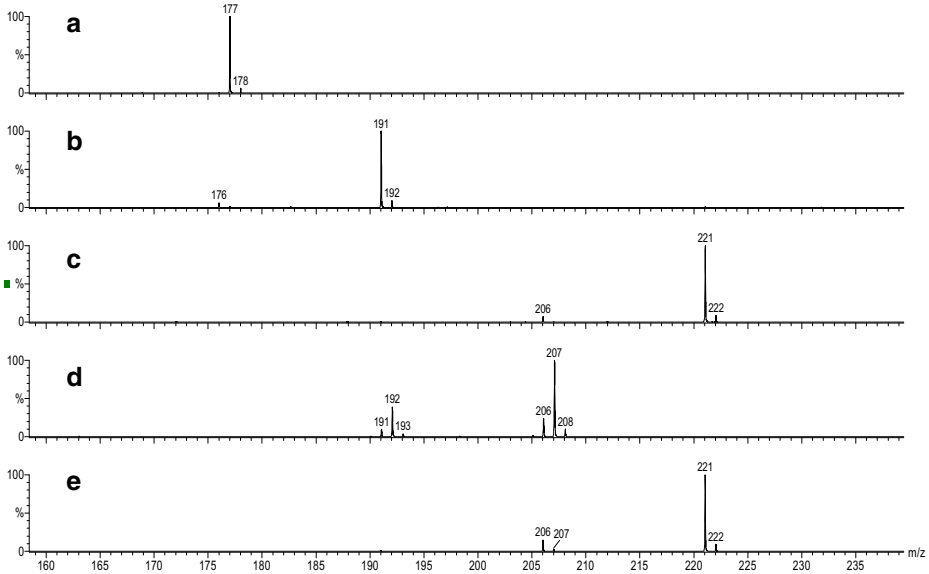


Fig. 3 Negative ion ms^2 spectra of the following hydroxycoumarin hexosides: **a** $[M\text{-}H]^-$ ion at *M/Z* 339 (compound 5), **b** $[M\text{-}H]^-$ ion at *m/z* 353 (compound 8), **c** $[M\text{-}H]^-$ ion at *m/z* 383 (compound 12), **d** $[M\text{-}H]^-$ ion at *m/z* 369 (compound 14), **e** $[M\text{-}H]^-$ ion at *m/z* 383 (compound 15)

was consistent with a previous study that failed to detect esculin, esculetin, chicoriin, or fraxin in the foliage of green or white ash (Santamour 1981). The occurrence of hydroxycoumarins and oleoside-type secoiridoids has been considered a characteristic feature of *Fraxinus* species (Iossifova et al. 1997; Jensen et al. 2002; Perez et al. 2005). In terms of phylogeny, our results suggest that green and white ash are chemotaxonomically distinct from Manchurian ash. This finding is supported by the molecular classifications of Wallander (2001) that placed green and white in the subsection *Melioides* (Endl.) Lingelsh, while Manchurian ash was placed in the subsection *Fraxinus*.

The majority of hydroxycoumarins and lignans we detected in the phloem of Manchurian ash have been previously reported from this species (Terazawa and Sasaya 1970; Tsukamoto et al. 1984, 1985). Compounds that were identified for the first time in this species included calceolariosides A and B, ligustroside, oleuropein hexoside, nuzhenide, tyrosol hexoside, and verbascoside. These compounds have been found in manna ash, which is endemic to the Mediterranean region and south-central Europe (Kostova 2001).

In terms of biological activity, hydroxycoumarins have been correlated with deterrence of insect feeding (Leszczynski et al. 1995). For example, in common annual sunflower (*Helianthus annus* L.), the induced synthesis of two coumarins, ayapin and scopoletin, was identified as one component of a defensive strategy against sunflower beetle (*Zygogramma exclamationis* F.) (Roseland and Grosz 1997). Silva et al. (2006) suggested that many plant β-glucosides, including esculin may impair insect development by inhibiting trehalase, which is a key enzyme controlling glucose availability in insects. We also detected the phenylethanoids, calceloariosides A and B, only in Manchurian ash, but their bioactivity has not been examined. However, another phenylethanoid, verbascoside, had no negative effects on the growth of buckeye butterfly (*Junonia coenia* Hübner) on *Plantago lanceolata* L. (Adler et al. 1995).

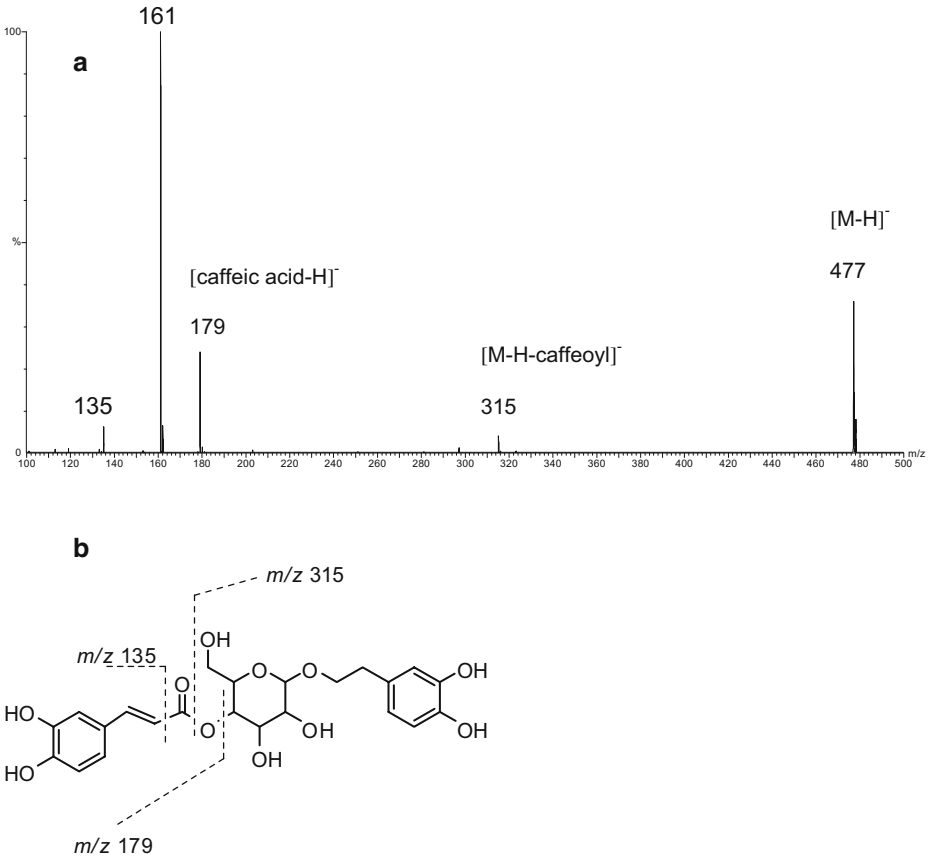


Fig. 4 **a** Negative ion ms^2 spectrum of compound 27; **b** proposed fragmentation pattern for $[M\text{-}H]^-$ ion 477

Secoiridoid glycosides were detected in all three species. As the name implies, the secoiridoids are structurally related to iridoids (cyclopentanoid monoterpenes), an important class of secondary defensive plant compounds, which have been implicated in plant–herbivore interactions (Nieminen et al. 2003; Biere et al. 2004; Harvey et al. 2005; Penuelas et al. 2006). According to Konno et al. (1999, 2001), secoiridoid glycosides such as oleuropein have anti-feedant properties based on their strong protein-complexing activity after hydrolysis by plant β-glucosidases. Biere et al. (2004), however, proposed that for the beet armyworm (*Spodoptera exigua* Hübner), iridoid glycosides may directly act as feeding deterrents rather than post-ingestive inhibitors, due to their extremely bitter taste.

In the present study, pinoresinol glucoside and pinoresinol dihexoside were detected in Manchurian ash in significantly higher amounts than in green or white ash. Structurally, pinoresinol is one of the simplest lignans, being a dimer of two coniferyl alcohol units. Lignans consist of dimers of phenylpropanoid (C6–C3) units enzymatically coupled through linkages between the propane chains (Willfor et al. 2003). A number of lignans have been reported to be insecticidal. For example, pinoresinol has antifeedant activity and inhibits larval growth and molting of the bloodsucking bug (*Rhodnius prolixus* Stal) and the milkweed bug (*Oncopeltus fasciatus* Dallas) (Cabral et al. 1999; Garcia et al. 2000). More

Table 4 Contents[a] of selected individual phenolic compounds in green, Manchurian, and white ash

Number	Compound	Green	Manchurian	White
38	Apigenin	0.08±0.01 a	ND[b]	0.30±0.02 b
27	Calceolarioside A	ND	12.4±1.95	ND
35	Calceolarioside B	ND	4.71±0.60	ND
7	Esculetin	ND	0.46±0.06	ND
5	Esculin	ND	0.44±0.01	ND
14	Fraxin	ND	14.9±0.60	ND
16	Fraxetin	ND	2.25±0.10	ND
12	Fraxidin hexoside	ND	7.24±0.76	ND
8	Methylesculin	ND	0.46±0.02	ND
15	Mandshurin	ND	19.8±1.43	ND
36	Ligustroside	11.2±0.68 ab	9.95±0.73 a	13.1±0.85 b
22	Oleuropein	3.62±0.52 a	–[c]	2.23±0.43 a
29	Oleuropein hexoside	8.76±0.64 a	1.34±0.06 b	8.72±0.55 a
19	Pinoresinol dihexoside	1.67±0.10 a	19.6±1.84 b	1.92±0.23 a
26	Pinoresinol glucoside	4.75±0.65 a	13.8±1.17 b	6.45±0.70 a
28	Syringaresinol	14.6±1.01	ND	12.7±0.87
32	Verbascoside	1.81±0.25 a	0.45±0.07b	4.00±0.69 c

Values in each row followed by different letters are significantly different ($P<0.05$).

[a] Contents expressed in milligrams per gram of fresh weight ($n=8\pm$SE)

[b] *ND* Not detected

[c] Detected by LC-MS but not LC-UV

recently, pinoresinol sequestered from its host plant was found in the defensive secretion produced by glandular hairs of cabbage butterfly caterpillars (*Pieris rapae* L), where it acts as a potent antifeedant to ants (*Formica exsectoides* Forel) (Schroeder et al. (2006).

In summary, we have documented substantial qualitative and quantitative differences between the constitutive phloem chemistry of Manchurian ash, which is resistant to EAB, and North American ash species, which are highly susceptible. Many of the compounds unique to Manchurian ash, or present in higher concentrations, are toxic or deterrent to other herbivores. However, *in vitro* and/or *in vivo* insect bioassays will be required to determine whether any of these actually contribute to EAB resistance of Manchurian ash. While our study focused on constitutive phloem chemistry, induced phytochemical responses to stem invasion are critical components of tree defense to beetles and pathogens (e.g., Bostock and Stermer 1989; Raffa and Klepzig 1992; Franceschi et al. 2005) and may also contribute to resistance of deciduous trees to wood-borers. As such, this study also provides a starting point for investigations of feeding-induced responses of ash to EAB.

Acknowledgements Thanks to Duan Wang for technical assistance. Use of the 400 MHz NMR spectrometer was permitted by the Ohio State University College of Pharmacy. We thank an anonymous reviewer for helpful comments. This work was supported in part by The Ohio Plant Biotechnology Consortium (Award No. 2005-025 to DAH, PB, and DFC), USDA Forest Service North Central Research Station (Award No. 03-JV-90 to DAH), USDA Northeastern Research Station (Research Joint Venture No. 05-JV-11242328-091 to DAH and PB), USDA ARS (Agreement No. 58-3607-4-175 to DAH), and by State and Federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

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Introduction

Goldenseal (*Hydrastis canadensis* L., Ranunculaceae) is an herbaceous perennial that is found in North American under mesic cove forest canopies throughout much of the south and eastern seaboard and north into Canada. The rhizome, rootlets, and root hairs produce medicinally active alkaloids, including the major alkaloids berberine, canadine, and β-hydrastine. Because of the alkaloids, *Hydrastis* is an important dietary supplement, which currently retails at a price of over $160 per pound. Indigenous use of *Hydrastis* by native Americans was widespread for a variety of infectious diseases (Moerman 1986). Today, the rhizome and rootlets are used as an antibacterial treatment, for skin infections, conjunctivitis, otitis media, and urinary tract infections. However, the plant has been aggressively harvested from its native setting (wildcrafted) and is now listed in Appendix II of the Convention on International Trade in Endangered Species (CITES). Because of harvesting pressures and loss of habitat, increasingly more of the raw material for medicinal plant use originates from cultivated sources rather than from wildcrafted plants. Our understanding of reciprocal rhizosphere influence on formation of clinically relevant plant secondary metabolites is woefully inadequate. We have little data on how fungi in the plant root rhizosphere may alter the ratios and makeup of medicinally important plant metabolites or whether those same plant compounds present in root exudates have a selective effect on the composition of the fungal rhizosphere community. Although individual alkaloids, berberine and hydrastine, have been reported to have antifungal activity (Cernakova and Kostalova 2002; Goel et al. 2003), neither *Hydrastis* alkaloids in combination nor the *Hydrastis* whole root extract have been investigated for effect on fungi. This study explores whether the level of association between *Fusarium* and *Hydrastis* can be distinguished by the fungal responses to alkaloid treatments and if the treatment effects are the products of additive or synergistic interactions of two or more *Hydrastis* alkaloids.

Materials and Methods

Fungal Isolation Three media were prepared, Czapeks Dox containing penicillin G (10,000 IU/ml) and streptomycin (10,000 μg/ml), water agar containing the same antibiotics, and carnation leaf agar (Sigma, St. Louis, MO, USA). Wildcrafted and commercial *H. canadensis* seeds (Horizon Herbs, Williams, OR, USA) were sterilized in 10% Clorox and plated on media. After 5 d at room temperature, transfers were made to potato dextrose agar (PDA), and after 7–10 d, the fungi were identified. *Hydrastis canadensis* leaf, stem, rhizome, and rootlet tissue were surface sterilized with 10% Clorox and plated on water agar. Isolates were continuously transferred until pure cultures were growing. *Fusarium* cultures were identified by the *Fusarium* Research Center, Department of Plant Pathology, Pennsylvania State University (PSU). By using a modified staining method (Koske and Gemma 1989), *H. canadensis* root and seed tissue was examined directly for arbuscular mycorrhizae (AM). To detect filamentous, non-AM fungal growth *in situ*, root tissue was dipped in 95% ethanol and flamed briefly. Root tissue was cut into 5-mm sections, and seeds were stripped of their pericarp. Lactophenol blue was added to each tissue, and tissue were examined microscopically (×40). *Hydrastis canadensis* soil samples were collected from soil adhering to the rootlets of *H. canadensis* plants and from a site devoid of *H. canadensis* or any other herbaceous plant. Both soils were screened also for the presence of AM reproductive spores by using screens of increasingly smaller mesh. In addition, soil slurries were prepared and added to the three isolation media. After 3 d, fungi were transferred to PDA

tubes and identified. Corn kernels were added directly to both soil samples to isolate pathogenic fungi. After 14 d, kernels were plated on water agar containing antibiotics. Fungal transfers were made.

Stock Hydrastis Extraction and Standards *Hydrastis canadensis* tissue was air-dried and ground (20 mesh) with a Wiley mill. Root and rhizome samples (100 mg) were defatted with 50 ml *n*-heptane for 2 h on a Labline Orbital Shaker at 100 rpm in dark brown bottles. Samples were filtered (Whatman #1) and then dried for 2 h. Fifty ml 50% methanol: 1% acetic acid were added, and samples were placed on a shaker (100 rpm), 25°C, 24 h. The sample was filtered and re-extracted twice. The three filtrate samples were combined, filter sterilized (Nalgene 0.45 μm), evaporated, and stored in a dark bottle at 5°C. A voucher specimen of *H. canadensis* (#05001ARZ) is stored in the Norton Brown Herbarium, University of Maryland, College Park, MD, USA. Alkaloid standard stock solutions (*w/v*) of 0.12% berberine dihydrochloride (Chromadex, Santa Ana, CA, USA), 0.02% canadine (Chromadex, Santa Ana, CA, USA), 0.08% hydrastinine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were prepared and filter sterilized.

Spore Counts Seven treatments were added in 1-ml increments to 125-ml Erlenmeyer flasks wrapped in aluminum foil: three individual alkaloid standards, all three alkaloids combined into a standard mix, a 0.2% (dry *w/v*) *Hydrastis* root extract (05001ARZ), a control of 50% methanol (1% acetic acid), and water. A spore suspension of three *Fusarium* isolates 5001AR (*Fusarium oxysporum*, from *Hydrastis* rootlet tissue), PSU 0-1174 (*Fusarium commune*, from PSU) and 9001ARC (*Fusarium solani*, from *Hydrastis* rhizosphere soil) was prepared. Inoculated carnation leafs were added to distilled water, filtered through sterile cheesecloth, and resuspended in 0.1% Tween 20. One milliliter (10^6 conidia) of each isolate was added to flasks containing treatments and incubated in 25 ml of Czapeks Dox medium for 0,1,2,3, and 4 d. Hyphae were filtered, lyophilized, and dry weight was determined. Filtrate was also collected, lyophilized, and stored at 5°C. Chlamydospore formation and macroconidia germination rates from lyophilized, 4-d-old mycelia were determined with a Bright line hemocytometer and light microscopy (×40).

Mycotoxin Analysis Freeze-dried filtrates were analyzed by using enzyme-linked immunosorbent assay (ELISA; Veratox, Neogen, Lansing, MI) to determine if *Hydrastis* alkaloids altered *Fusarium* mycotoxin production.

Statistical Analysis The data of isoquinoline alkaloid treatment effects on the fungal response, including macroconidial germination and mycotoxin production, were normally distributed; therefore, they were analyzed with a paired *t*-test.

Results and Discussion

Results from screening *Hydrastis* tissue and surrounding soil provide limited evidence of a selective rhizosphere effect. From native *Hydrastis* plant populations, *F. oxysporum* was isolated from disease-free rootlet and seed tissue, whereas *Pythium* spp. was isolated from seed. The *F. oxysporum* isolate was microscopically detected intracellularly and localized near rootlet tips. In addition, coenocytic hyphae were also detected in the same *Hydrastis* root pericycle tissue from which *F. oxysporum* (05001AR) isolation occurred, although the

unknown fungus was never isolated in culture. Microscopic examinations of stained *Hydrastis* root tissue provided no evidence for AM fungi, nor were AM chlamydospores found in any of the soil collections. Mesic cove forests where *Hydrastis* was collected are not particularly conducive to high populations of AM fungi. *Fusarium solani* and *Pythium* sp. were present in *Hydrastis* rhizosphere soil. Species isolated from non-rhizosphere soil include *Rhizoctonia solani* and *Gliocladium* spp. Isolates from commercial *Hydrastis* tissue include *F. solani* from rootlet and leaf tissue, and *F. oxysporum* isolated from seed. No fungi were isolated from rhizome or stem tissue of any *Hydrastis* sample. Although the *Hydrastis* rhizosphere soil does not drastically alter the fungal community, the presence of *F. oxysporum* within *Hydrastis* root tissue suggests that elements of *Hydrastis* root exudates may influence the level of association of *Fusarium* species with root tissue.

To model *Hydrastis/Fusarium* interactions at the host plant root interface, an assay was designed based on the assumptions that berberine, canadine, and hydrastine are present in *Hydrastis* root exudates, as evidenced by plant cell culture experiments (Maddox et al. 1999). The whole root extract was chosen to model the complex of compounds present in *Hydrastis* root exudates. Analysis with liquid chromatography-mass spectroscopy (data not shown) indicated that the whole root extract contained berberine, canadine, and hydrastine. Based on previous *Hydrastis* extraction studies (Galeffi et al. 1997; Gentry et al. 1998; McNamara et al. 2004), minor alkaloids and non-alkaloid components should also be present. Three *Fusarium* species exhibiting different levels of association with *Hydrastis* root tissue and rhizosphere were identified. Treatment effects on *Fusarium* growth and spore formation were not significant for the three species (data not reported). However, macroconidia germination rates represented a significant response factor, as summarized in Fig. 1. All individual alkaloid treatments significantly inhibited *F. oxysporum* macroconidia germination (75–100%), whereas the whole root extract treatment stimulated germination (25%). *Fusarium solani* germination was significantly stimulated by the whole root extract (200%), whereas only the alkaloid standard treatment inhibited spore germination (75%). In contrast, the germination rate of the non-*Hydrastis* isolate, *F. commune*, was inhibited by all treatments; especially by the whole root extract treatments (40%). The components responsible for the inhibition of macroconidia germination of the *Hydrastis* isolates appear to be berberine and canadine. None of the major alkaloid compound(s) found in the whole root extract stimulated any isolate, whereas only the *Hydrastis* isolates were stimulated by the whole root extract. The results suggest that non-alkaloid components and/or minor alkaloids were stimulatory singly or synergistically, although additional experiments are required to be certain of the active component. Because disease symptoms were absent in the *Hydrastis* root tissue from which the *F. oxysporum* was isolated, this suggests that the isolate is nonpathogenic and may be endophytic. Gunawardena et al. (2005) noted that *Pisum sativa* (pea) root exudates stimulated macroconidia germination in *F. solani* species complex (*Nectria haematococca*) in a host-specific pattern and influenced the establishment of the endophytic relationship. Although the *F. commune* isolate exhibited the highest macroconidia germination rates, only the *Hydrastis* isolates responded positively to the whole root extract treatment by increasing germination rates. Growth within *Hydrastis* root tissue occurred only with the *F. oxysporum* isolate, suggesting that signal compounds present in the extract may be influencing the endophytic status.

Changes to the cultural media pH can influence fungal response. Both *F. commune* and *F. solani* exhibited a similar pattern of pH change with treatments, except that the changes in the pH values for *F. commune* whole root extract and methanol treatments lagged slightly (data not shown). The change in pH values for *F. oxysporum* whole root extract and methanol treatments were similar to the *F. commune* isolate. However, the initial pH values

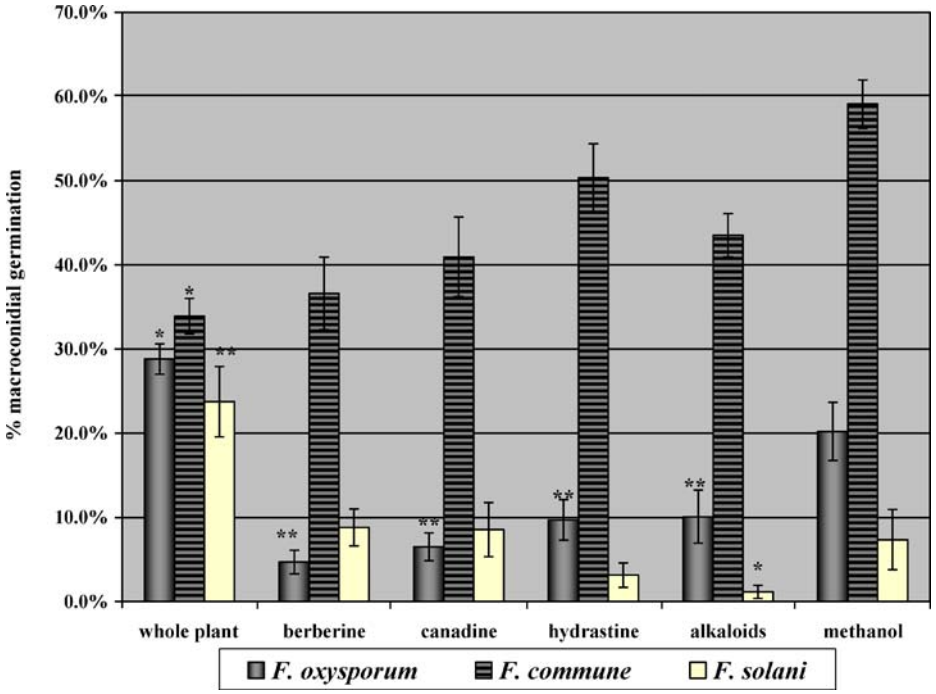
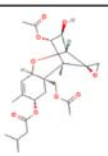
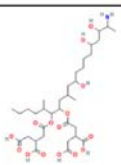
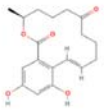


Fig. 1 Isoquinoline alkaloid effect on germination of macroconidia by three *Fusarium* spp. grown in Czapeks Dox liquid media. Treatments—berberine 0.12 mg ml−1, canadine 0.02 mg ml−1, hydrastine 0.08 mg ml−1, and whole root extract 2.00 mg ml−1—were dissolved in 1 ml of 50% methanol/1% acetic acid solvent and added to 25 ml of culture medium containing 1.0 ml fungal inoculum (approx. 106 conidia ml−1). Samples (N=12) expressed per mg of dry weight mycelia on d 4 of the assay (*error bars* the standard deviation of the error, *P<0.05, **P<0.01, paired t-test)

of individual alkaloid treatments were higher than the other isolates (pH 6.5 vs. pH 4.75), and remained fairly constant during the study. It is unclear which element was buffering the media—the basicity of alkaloid treatments or the response of the endophytic isolate. In either case, treatment replicates were randomized with respect to both fungus and treatment, which suggests that the pH pattern exhibited is unique to the endophytic isolate and is not because of differences in pH between alkaloid medium and the control.

Production and excretion of mycotoxins might also play a role during interactions at the rhizoplane. All *Fusarium* isolates were screened for mycotoxins at the end of the growth phase on d 4 (Table 1). Ueno et al. (1977) reported that the *F. commune* isolate was capable of producing the mycotoxin zearalenone (ZON) in culture. The results indicate that the mycotoxin was present in the water treatment during our assay. All other treatments, particularly berberine, canadine, and whole root extract, inhibited the production of ZON in the *F. commune* isolate, which did not produce any other mycotoxin. The *F. solani* isolate did not produce detectable mycotoxin levels in any treatment. All alkaloid treatments were inhibitory to fumonisin production, especially berberine. Canadine significantly stimulated T-2 mycotoxin production by the *F. oxysporum* endophyte, whereas hydrastine had less of a stimulatory effect. The alkaloid standard treatment significantly stimulated production of ZON by *F. oxysporum*. The whole root extract, canadine, and methanol treatments had no effect, whereas berberine and hydrastine treatments had an inhibitory effect on ZON production. Because the ratios of major alkaloids were approximately equal between the

Table 1 Mycotoxin production by *Fusarium* in media containing isoquinoline alkaloids

Fungus	Treatment	Mycotoxin		
		T-2	fumonisin	ZON
				
F. oxysporum				
	whole root extract	0.05	0.00*	1.95
	berberine	0.06	0.00*	0.00*
	canadine	0.24*	1.05	1.87
	hydrastine	0.11	3.98	0.00*
	alkaloid mix	0.04	3.17	7.61*
	methanol	0.00	5.77	3.03
	water	0.00	3.19	0.00
F. commune				
	whole root extract	0.00	0.00	0.00*
	berberine	0.00	0.00	0.00*
	canadine	0.00	0.00	0.00*
	hydrastine	0.00	0.00	1.07
	alkaloid mix	0.00	0.00	2.32
	methanol	0.00	0.00	1.30
	water	0.00	0.00	6.57
Correlation coefficient of calibration curves ($P < 0.01$)		0.9863	0.9984	0.9996

The data for this graph was collected from the same experiment as Fig. 1. Sample measurements ($N=3$) were taken of mycelia filtered from culture on d 4 using Neogen ELISA fluorescence plate reader. Results expressed as ng metabolite/mg dry weight mycelia.

*$P<0.05$, paired *t*-test

whole root extract and alkaloid standard treatments, the results suggest a synergistic effect among the three alkaloids that was able to overcome the inhibitory effects of berberine and hydrastine in the alkaloid standard treatment but not in the whole root extract treatment. It is possible that minor alkaloids or non-alkaloid components of the whole root extract prevented the synergistic interaction. Overall, mycotoxin mass values were near the limits of detection for the Neogen ELISA kit used in this study. However, preliminary screening indicated that the *F. oxysporum* endophyte is metabolically more active in producing mycotoxins and, thus, may be more competitive with other rhizosphere fungi at the *Hydrastis* rhizoplane.

As cultivated *Hydrastis* roots are used more frequently for the manufacture of commercial herbal medicine rather than wildcrafted sources, future work is required to understand if the endophytic status of *F. oxysporum* alters the alkaloid content and ratios of *Hydrastis* root tissue. This study provides the first evidence that medicinally active *Hydrastis* alkaloids influence *Fusarium* isolates in the rhizosphere.

Acknowledgements I acknowledge Paul Strauss, for permission to wildcraft goldenseal on United Plant Savers land and Alex Johnson for contributions to the ELISA detection. This study is part of the Ph.D. dissertation research undertaken by Michael C. Tims.

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For a fast and reliable bioassay, the test plant species must germinate uniformly and grow relatively quickly (Dayan et al. 2000). Increasingly, plant biologists have been turning to *Arabidopsis*, the favored system for the study of plant molecular genetics and development (Mitchell-Olds 2001; Shimizu and Purugganan 2005). Specifically, *Arabidopsis thaliana* (L.) Heynh. is a useful test species because it shows uniform germination and rapid growth, and thus represents an excellent model for the study of plant responses to allelochemicals and other environmental toxins (Baerson et al. 2005).

In this study, we selected two response parameters, seed germination and root radicle elongation, to obtain preliminary assessments of the activity of 21 plant secondary metabolites commonly considered as natural phytotoxins (Reigosa et al. 1999). *Arabidopsis thaliana* was used as plant model. Dose–response curves were obtained for each test compound, allowing the determination of several key parameters including the inhibition threshold (lowest concentration required to initiate inhibition), the IC_{50} (concentration required to obtain 50% inhibition), and the lowest complete inhibition concentration (LCIC) for each effect. These parameters are important because they establish a frame of reference to which subsequent tests can be compared (Dayan et al. 2000).

Methods and Materials

Three experiments were performed. The first evaluated the effects of the different compounds on germination. The other two evaluated effects on root elongation in the presence and absence of nutrient solutions.

Chemicals

Twenty-one molecules were tested, all obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of protocatechualdehyde (Merck, Germany). Stock solutions of test compounds were made in different solvents based on the compound's solubility: 1 M ethanol for eucalyptol, quercetin, vanillin, sinapinic acid, syringic acid, syringaldehyde, vanillic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, and rutin; 1 M dimethyl sulfoxide for gallic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid, *trans*-cinnamic acid, 2-benzoxazolinone (BOA), protocachualdehyde, and tyrosol; and 2×10^{-3} M distilled water for the less soluble juglone and L-mimosine (see Fig. 1 for all structures).

From these stocks, various aqueous solutions were made up and adjusted to pH 6 with NaOH (Jose and Gillespie 1998; Martin et al. 2002): 10, 50, 100, 500, 750, and 1,000 μM for the germination test and root elongation without nutrients test, and 125, 250, 500, and 1,000 μM for the root elongation with nutrients test. In all experiments, a control with distilled water and another control with the solvent were prepared.

Seeds

Seeds of *A. thaliana* L. (Heynh.), ecotype Columbia, obtained from Herbiseed, were surface-sterilized as described in Baerson et al. (2005).

Bioassays

For germination tests and root elongation tests without nutrients, seeds were placed in 24-well plates with 1.25-cm-diam Whatman no.1 paper disks (Maidstone, England), and

Gallic acid	R1: COOH	R2: H	R3: OH	R4: OH	R5: OH	R6:H
Protocatechuic acid	R1: COOH	R2: H	R3: OH	R4: OH	R5: H	R6:H
Gentisic acid	R1: COOH	R2: OH	R3: H	R4: H	R5: OH	R6:H
p-Hydroxybenzoic acid	R1: COOH	R2: H	R3: OH	R4: H	R5: H	R6:H
Syringic acid	R1: COOH	R2: H	R3: OCH_3	R4: OH	R5: OCH_3	R6:H
Vanillic acid	R1: COOH	R2: H	R3: OCH_3	R4: OH	R5: H	R6:H
Transcinnamic acid	R1: (CH_2)COOH	R2: H	R3: H	R4. H	R5: H	R6: H
p-Coumaric acid	R1: (CH_2)COOH	R2: H	R3: OH	R4. H	R5: H	R6: H
Sinapinic acid	R1: (CH_2)COOH	R2: H	R3: OCH_3	R4: OH	R5: OCH_3	R6: H
Ferulic acid	R1: (CH_2)COOH	R2: H	R3: OCH_3	R4: OH	R5: H	R6: H
Chlorogenic acid	R1: (CH_2)COO-R	R2: H	R3: OH	R4: OH	R5: H	R6:H
Protocatechualdehyde	R1: CHO	R2: H	R3: OH	R4: OH	R5: H	R6: H
Syringoaldehyde	R1: CHO	R2: H	R3: OCH_3	R4: OH	R5: OCH_3	R6: H
Vanillin	R1: CHO	R2: H	R3: OCH_3	R4: OH	R5: H	R6: H

BOA	$C_7H_5NO_2$
Juglone	$C_{10}H_6O_3$
L-Mimosine	$C_8H_{10}N_2O_4$
Quercetin	$C_{15}H_{10}O_7$
Rutin	$C_{27}H_{30}O_{16}$
Tyrosol	$C_8H_{10}O_2$
Eucalyptol	$C_{10}H_{18}O$

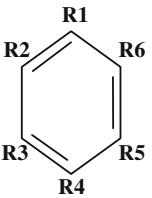


Fig. 1 Structures of the compounds studied

watered with 200 μl of the test solution or control. All control and test treatments were replicated three times for germination tests, and eight times with 8 pregerminated seeds, for root elongation tests without nutrients. For root elongation with nutrients supplied, 25 seeds were placed in square petri plates with a semisolid media containing 0.5× Murashige and Skoog salts, 1× Gamborg's B5 vitamins, 1% sucrose (w/v), and 2% Gelrite® (w/v) adjusted to pH 6. All treatments and controls were replicated four times.

Plates were placed in a cold chamber for 3 d at 4°C to synchronize germination. Plates with cold stratified seeds were then transferred to a growth chamber with day/night temperatures of 22 and 20°C, respectively. Average light intensity was maintained at ~80 μmol m^{-2} sec^{-1} during a 16-hr light cycle.

Germination was assessed at 0, 12, 24, 36, and 48 hr with a magnifying glass. The data were used to calculate the germination index G_T, and all alternative indices (*S*, speed of germination; AS, speed of accumulated germination; CRG, coefficient of rate of germination; and MGT, mean total germination) as described in Chiapusio et al. (1997).

Before measurement of roots, plates were frozen at −18°C. Radicle length was measured 6 d after the beginning of the experiment in the test without nutrients, and 14 d after in the test with nutrients.

Statistical Analysis

Mean germination and mean germination indices were compared to their respective controls by using the Mann–Whitney *U* test ($P<0.05$). All dose–response curves were modeled by regression analysis with SPSS® models (linear, logarithmic, inverse, quadratic, cubic, potential, growth, and exponential), and tested by using the Mann–Whitney *U* test ($P<0.05$) selected on the basis of the determination coefficient.

Results and Discussion

Germination Tests

The organic solvents used for dissolving the compounds had no significant effects, either in the germination or root elongation tests.

Total germination, G_T, is an overall assessment of the effect on germination (inhibition, stimulation, or no action) (Chiapusio et al. 1997). G_T was affected by more than half the molecules tested (*trans*-cinnamic, ferulic, *p*-hydroxybenzoic, sinapinic, *p*-coumaric, gentisic, and gallic acids, and protocatechualdehyde, vanillin, L-mimosine, and juglone).

trans-Cinnamic and ferulic acids inhibited *A. thaliana* total germination above 500 and 750 μM, respectively (Fig. 2). This inhibition is stronger than that reported by Li et al. (1993) for *Lactuca sativa* L., in which an inhibitory effect required a concentration greater than 10^{-3} M. Reigosa et al. (1999) found that these compounds have a stimulatory effect or no action on various weeds. In the present study, *p*-hydroxybenzoic acid was also inhibitory at 750 and 1,000 μM (Fig. 2). No actions of *p*-hydroxybenzoic and ferulic acids on *Poa annua* L. germination were reported by Wu et al. (1998). Gentisic acid and protocatechualdehyde had inhibitory effects on total germination only at 1,000 μM (Fig. 2). In contrast, Chiapusio et al. (1997) found no action of gentisic acid on *L. sativa* germination. The two molecules with the strongest inhibitory effects were L-mimosine and juglone; juglone totally inhibited germination at concentrations above 100 μM (Fig. 2). This compound, which has been previously reported as a potent allelochemical (Szabo 2000), also had effects on the other four germination indices (Table 1).

Four compounds—vanillic acid, L-mimosine, juglone, and *trans*-cinnamic acid—delayed *A. thaliana* germination at the highest concentration tested (Table 1). Juglone was the only compound for which the three dose–response curve parameters could be calculated. By using

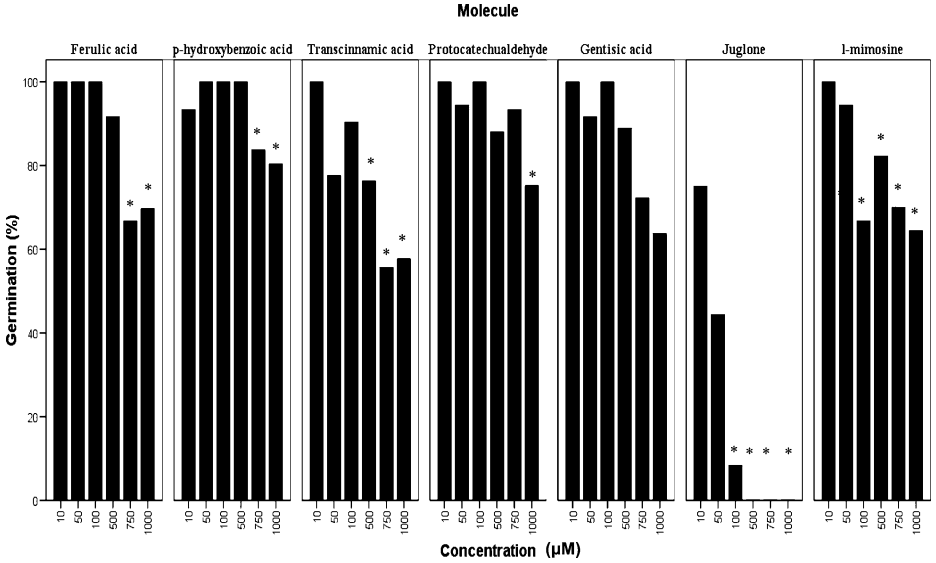


Fig. 2 Inhibitory effects of different concentrations (WM) of seven of the molecules assayed on total germination (%) of *A. thaliana*. *Indicates significant differences at the 0.05 level compared with control by Mann–Whitney *U* tests

a logarithmic adjustment: the inhibition threshold was ~20 μM, the IC_{50} ~40 μM, and LCIC ~100 μM. For the other compounds the highest concentration assayed was near the inhibition threshold.

Tyrosol, quercetin, BOA, rutin, eucalyptol, syringaldehyde, and syringic, and vanillic, protocatechuic, and chlorogenic acids had no significant effects on *A. thaliana* total germination (data not shown). Likewise Angelini et al. (2003) found that eucalyptol had no effect on the germination of three weed species.

Root Elongation Tests

Radicle length was inhibited by the most of the compounds tested both without nutrients (L-mimosine, juglone, syringaldehyde, protocatechualdehyde, vanillin, BOA, and *trans*-cinnamic, gentisic, gallic, syringic, vanillic, sinapinic, chlorogenic, ferulic, *p*-coumaric, protocatechuic, and *p*-hydroxybenzoic acids), and with nutrients (L-mimosine, juglone, syringaldehyde, protocatechualdehyde, vanillin, BOA, and *trans*-cinnamic, gallic, syringic, vanillic, sinapinic, chlorogenic, ferulic, *p*-coumaric, protocatechuic, and *p*-hydroxybenzoic acids).

Table 2 summarizes the inhibitory effects of the tested compounds on root growth of *A. thaliana* in the absence of nutrients, using the best-fit equation based on the coefficient of determination (r^2), and estimated values of IC_{50} and IC_{80}, for the 21 compounds assayed in the root elongation test without nutrient supply. Table 3 summarizes the inhibitory effects of the tested compounds on root growth in the presence of nutrients.

L-Mimosine and juglone had the strongest inhibitory effects on root growth of *A. thaliana* seedlings in the absence of nutrient solution (Table 2). Jose and Gillespie (1998) reported similar inhibition of root growth by these compounds in a study of corn and soybean.

Ferulic and *p*-coumaric acids likewise strongly inhibited root length in the absence of nutrients (Table 2). In contrast, Reddy and Rao (1999) found that ferulic acid improved tobacco growth whereas *p*-coumaric acid was inhibitory. This is line with the view that different plant species can vary enormously in their response to potentially allelopathic substances (Baleroni et al. 2000).

Table 1 Effects of various concentrations of juglone, L-mimosine, *trans*-cinnamic, and vanillic acids on germination indices (S, AS, CRG, and MGT)

Concentration (μM)	Molecule								
	Juglone				L-Mimosine		*trans*-Cinnamic Acid		Vanillic Acid
	S	AS	CRG	MGT	S	AS	S	AS	S
Control	55.6	123.7	3.3	111.8	55.6	132.7	55.6	132.7	155.6
10	42.6	106.8	3.3	113.3	57.6	135.7	61.1	145.0	51.9
50	40.4	97.1	3.1	95.6	47.2	113.8	49.5	122.9	51.8
100	5.6	9.9	0*	47.3*	46.6	118.9	55.8	135.0	69.1
500	0*	0*	0*	0 b	45.2	111.3	42.3	107.5	47.6
750	2.2*	2.2*	0*	16.0	4.8	108.2	35.9*	84.8*	45.5
1,000	0*	0*	0*	0*	32.7*	90.3*	32.4*	83.7*	40.2*

*Indicates significant differences at the 0.05 level compared with control (Mann–Whitney *U* tests)

S = Speed of germination, AS = speed of accumulated germination, CRG = coefficient of rate of germination, MGT = mean total germination

Table 2 Regression analyses of dose–response curves for effects on *A. thaliana* growth of different concentrations of the 21 compounds assayed in the absence of nutrients

Molecule	Regression Equation	r^2	IC_{50} (μM)	IC_{80} (μM)
L-Mimosine	$y = 5.42 + 173.16/x$	0.745	3.9	11.9
Juglone	$\ln(y) = 1.43 + 17.73/x$	0.847	7.1	11.3
trans-Cinnamic acid	$y = 398.69\ x^{-0.6847}$	0.935	20.7	79.1
Gentisic acid	$y = 611.10\ x^{-0.7305}$	0.891	30.8	107.9
Gallic acid	$y = 709.34\ x^{-70.58}$	0.832	42.8	156.9
Syringic acid	$y = 60.02e^{-0.0032x}$	0.854	57.1	343.4
Vanillic acid	$y = -19.11\ \ln(x) + 130.77$	0.677	69.8	337.6
Sinapinic acid	$y = -20.29\ \ln(x) + 141.23$	0.890	91.5	404.1
Chlorogenic acid	$y = 67.39e^{-0.0031x}$	0.912	96.3	391.8
Ferulic acid	$y = 77.11e^{-0.0036x}$	0.911	120.3	374.9
p-Coumaric acid	$y = 79.06e^{-0.0035x}$	0.939	143.2	392.7
Syringaldehyde	$y = 73.45e^{-0.0023x}$	0.884	167.2	565.5
Vanillin	$y = -19.34\ \ln(x) + 144.82$	0.880	172.6	622.4
Protocatechuic acid	$y = 89.77e^{-0.0032x}$	0.858	182.9	469.2
p-Hydroxybenzoic acid	$y = 97.97e^{-0.0035x}$	0.908	192.2	453.9
BOA	$y = 98.80e^{-0.0011x}$	0.689	619.1	1,452.1
Protocatechualdehyde	$y = -0.0574\ x + 91.39$	0.527	721.1	1,243.7
Eucalyptol	$y = 0.034\ x + 66.38$	0.488	N.I.	N.I.
Rutin	$y = 8 \cdot 10^{-8}x^3 + 10^{-4}x^2 - 0.05x + 69$	0.189	N.I.	N.I.
Tyrosol	$y = -2 \cdot 10^{-7}x^3 + 4 \cdot 10^{-4}x^2 - 0.3x + 118$	0.367	N.I.	N.I.
Quercetin	$y = 3 \cdot 10^{-8}x^3 + 10^{-5}x^2 - 0.03x + 80$	0.093	N.I.	N.I.

r^2 = Determination coefficient, IC50 = concentration required to obtain 50% root growth inhibition, IC80 = concentration required to obtain 80% root growth inhibition, N.I. = not inhibitory

Vanillic, *p*-coumaric, ferulic, and *p*-hydroxybenzoic acids inhibited radicle elongation in the test without nutrients (Table 2 and Fig. 3). Janovicek et al. (1997) reported similar findings with wheat seedlings, especially above 10^{-4} M.

In the test with nutrient solution, the order of phytotoxic effects of the different compounds changed (Table 3). The most active compound was protocatechualdehyde, whereas L-mimosine and juglone had less marked effects.

Nutrient limitation had a synergic effect with phytotoxicity. In the presence of nutrients, a higher concentration was necessary to obtain a reduction of 50% in root growth than without nutrients. This observation is in agreement with the results of Belz and Hurle (2004), who found that nutrient reduction increased the inhibitory effects of BOA. Availability of macronutrients like nitrogen is, along with light, the most important parameter in seedling growth and development. Martin et al. (2002) demonstrated that *A. thaliana* is phenotypically, physiologically, and metabolically affected by nitrogen deficit. Filleur et al. (2005) showed that *A. thaliana* has a box gene related to the modulation of root growth in response to the external supply of nitrogen. Even limitations in phosphate reduce root growth (Chevalier et al. 2003; López-Bucio et al. 2002). Thus, the observed effects in the experiment without nutrient solution could be explained by the lack of nitrogen and phosphate.

Four compounds—eucalyptol, rutin, tyrosol, and quercetin—did not show any appreciable effect on root growth (data not shown). Fig. 3 shows dose–response relationships for 6 of the remaining 17 tested compounds (juglone, protocatechualdehyde, and syringic, gallic, ferulic, and *p*-coumaric acids). A comparison of the structures and inhibitory effects of these six

Table 3 Regression analyses of dose–response curves for effects on *A. thaliana* growth of different concentrations of the 21 compounds assayed in the presence of nutrients

Molecule	Regression Equation	r^2	IC_{50} (μM)	IC_{80} (μM)
Protocatechualdehyde	$y = 92.82\ x^{-0.428}$	0.993	4.2	36.1
Juglone	$y = -0.0832\ x + 107.62$	0.962	692.9	1,053.1
BOA	$y = -0.0876\ x + 112.58$	0.988	714.4	1,056.8
Syringaldehyde	$y = 111.69e^{-0.0011x}$	0.976	730.6	1,563.6
Vanillin	$y = -0.087\ x + 115.08$	0.978	748.0	1,092.9
trans-Cinnamic acid	$y = -0.0586\ x + 98.078$	0.967	820.4	1,332.4
Gallic acid	$y = -0.0833\ x + 118.97$	0.952	827.9	1,188.1
Protocatechuic acid	$y = 106.31e^{-0.0009x}$	0.991	838.1	1,856.2
p-Hydroxybenzoic acid	$y = -0.069\ x + 111.5$	0.983	891.3	1,326.1
Syringic acid	$y = 109.01\ e^{-0.0008x}$	0.891	974.3	2,119.6
Vanillic acid	$y = -10^4x^2 + 0.16\ x + 66.1$	0.998	1,006.5	1,477.6
L-Mimosine	$y = -0.051\ x + 101.6$	0.976	1,007.1	1,598.8
Chlorogenic acid	$y = 103.21e^{-0.0007x}$	0.991	1,035.3	2,344.3
p-Coumaric acid	$y = -0.0622\ x + 114.58$	0.832	1,038.3	1,520.6
Sinapinic acid	$y = -0.046\ x + 98.88$	0.848	1,052.7	1,699.3
Ferulic acid	$y = -3 \cdot 10^{-4}x^2 + 0.32x + 60$	0.996	1,099.0	1,181.5
Eucalyptol	$y = -4.10^{-5}x^2 + 0.03x + 104$	0.569	N.I.	N.I.
Tyrosol	$y = 125.33\ e^{-0.0004x}$	0.894	N.I.	N.I.
Rutin	$y = -0.0185\ x + 101.28$	0.623	N.I.	N.I.
Gentisic acid	$y = 172.61\ x - 0.1122$	0.676	N.I.	N.I.
Quercetin	$y = -8 \cdot 10^{-5}x^2 - 0.08\ x + 96$	0.695	N.I.	N.I.

r^2 = Determination coefficient, IC50 = concentration required to obtain 50% root growth inhibition, IC80 = concentration required to obtain 80% root growth inhibition,. N.I. = not inhibitory.

compounds (Fig. 3) indicates that the most strongly inhibitory was the naphthoquinone juglone, followed by the hydroxybenzoic acids, gallic and syringic acids (Fig. 3 and Table 2), in which two phenolic hydroxy or methoxy groups (respectively) are substituted at ortho-positions to a phenolic hydroxyl group (Fig. 1). The hydroxycinnamic acids ferulic and *p*-coumaric acids, with only one hydroxy group, showed only weak inhibitory activity (Fig. 1). Of these six compounds, that showing the lowest inhibitory activity was the benzaldehyde protocatechualdehyde (Fig. 3).

In the absence of nutrients (Table 2 and Fig. 3), phenols that are structurally similar (Fig. 1) presented a range of phytotoxic effects. The strongest effect was produced by the molecules with acid radical (hydroxybenzoic acids), followed by the molecules with propenoic radical (hydroxycinnamic acids), and finally the molecules with aldehyde radical (benzaldehydes). In the presence of nutrients (Table 3 and Fig. 4), the molecules with aldehyde radical induced greater inhibition than the molecules with acid and propenoic radicals, in this order. Moreover, both in the presence and absence of nutrients the hydroxy radical had a greater effect than the methoxy radical.

In the present study, the hydroxybenzoic acids had a stronger effect than the hydroxycinnamic acids, which is in line with the results of Nakai et al. (2001). A possible explanation for this is that oxidative damage is greater in plants treated with hydroxybenzoic acids because they have less antioxidative effect. Another explanation could be that these compounds have more efficient interactions with cellular enzymes, but if so, the actual mechanism remains to be determined (Campos et al. 2003).

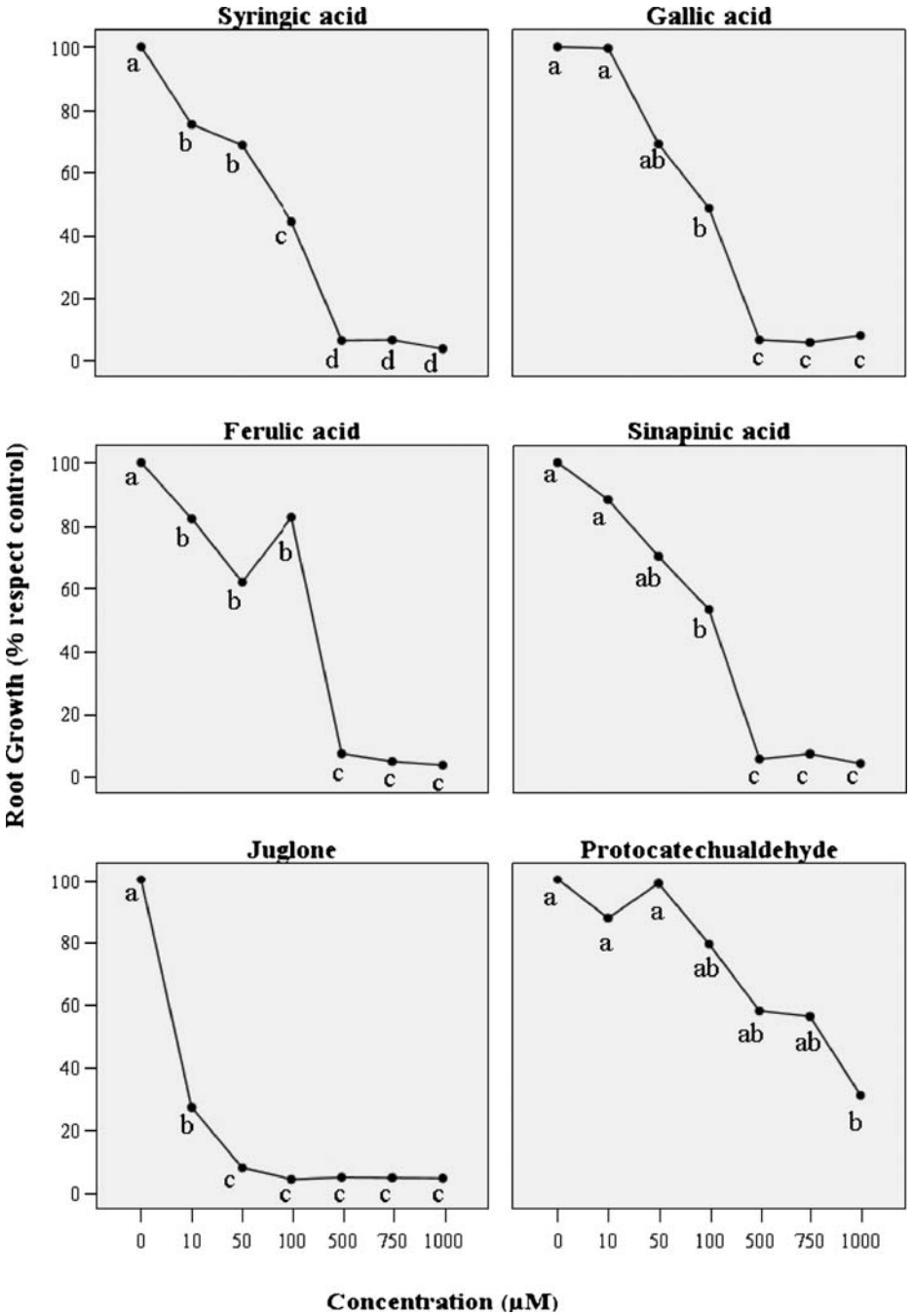


Fig. 3 Dose–response curves for root length growth (percentage with respect to control) in *A. thaliana* treated with six potential allelopathic compounds, at concentrations from 0 to 1,000 µM, without nutrients. Means with a different letter are significantly different ($P<0.05$, Mann–Whitney *U* test)

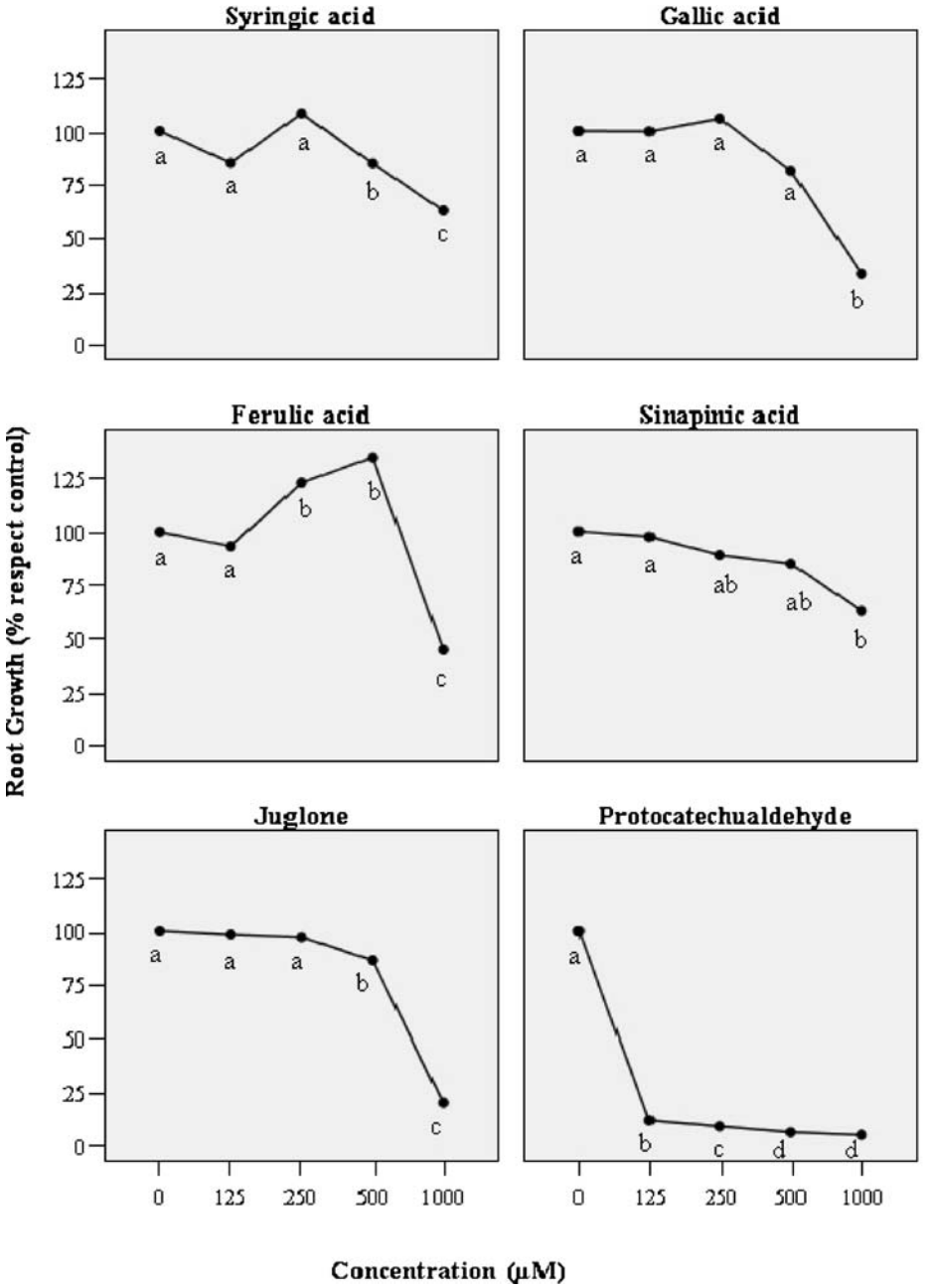


Fig. 4 Dose–response curves for root length growth (percentage with respect to control) in *A. thaliana* treated with six potential allelopathic compounds, at concentrations from 0 to 1,000 µM, with added nutrients. Means with a different letter are significantly different ($P<0.05$, Mann–Whitney U tests)

In summary, target species vary in their responses to phenolic compounds (Jose and Gillespie 1998; Dayan et al. 2000). In general, phenolic acids affect seedling growth more than germination (Reigosa et al. 1999). Hydroxybenzoic acids seem to be more toxic than the structurally similar hydroxycinnamic acids.

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Introduction

Ozone (O_3) is recognized as the most widespread and phytotoxic air pollutant in the northeastern part of North America (Skelly et al. 1997). Ozone is a strong oxidant, injuring plant tissues and disturbing the anatomical, biochemical, and physiological function of plants (Skäby et al. 1998; Kley et al. 1999). Also, it is known to affect carbon allocation and cause disturbance in carbon transport within and between plant organs (Laurence et al. 1994).

Plant secondary compounds in needles often deter herbivores and interfere with infections by fungi (Beckman 2000). Thus, changes in secondary metabolism as a result of gaseous air pollution are potentially important for the health of forests. Conifers produce oleoresin, a complex mixture of terpenoids, mainly composed of diterpene resin acids with minor amounts of monoterpenes and sesquiterpenes (Phillips and Croteau 1999). Oleoresin compounds act as feeding deterrents or stimulants to a wide variety of herbivores (Manninen 1999). Earlier studies have indicated slight O_3 effects on the concentrations of monoterpene (Kainulainen et al. 1998) and resin acids (Kainulainen et al. 1995, 2000) in conifers, whereas O_3 increased the concentration of total phenolic compounds (Rosemann et al. 1991; Booker at al. 1996). Another study, which examined pine (*Pinus* spp.) and spruce (*Picea* spp.) exposed to ozone and different nitrogen availability, concluded that elevated levels of O_3 do not affect the concentration of terpenoids from conifer stemwood (Manninen et al. 2002).

Lignin and other phenolic compounds in the foliage of plants may also be affected by O_3. Molecular and biochemical studies suggest that O_3 induces the synthesis of cinnamic acid derivatives via the shikimic and phenylpropanoid pathways (Rosemann et al. 1991; Eckey-Kalthenbach et al. 1994). Phenylpropanoid derivatives that are reported to accumulate in conifer needles in response to O_3 include hydroxycinnamic acids (Kicinski et al. 1988), flavonoids (Langebartels et al. 1990), and proanthocyanidins (Jordan et al. 1991). Lignin content in needles was not significantly affected by O_3 exposure although catechin (precursor to tannins) concentration increased with increased levels (Booker et al. 1996). One study examined the concentration of simple phenols in pine (*Pinus halepensis* Mill.) needles as bioindicators of NO, NO_2, and O_3 air pollution. Of the phenols studied, only vanillin decreased in concentration with measured increases in O_3.

The purpose of this study was to determine whether 2-yr-old white pine (*Pinus strobus* L.) foliage exposed to O_3 under field conditions affected the phenolic and resin acid concentrations in their needles. Comparatively little work has been done on this subject with *P. strobus*, a commercially important species in the Maritime provinces of Canada. Pine forest productivity is predicted to be reduced by current levels of ambient O_3 in the region (Charland et al. 1994).

Phenolic and resin acids were measured in the needles of clones originating from populations growing in two different regions with different air pollution levels. Profiling of the phenolic and acidic compounds was done directly on needles by using a relatively new and rapid method of analysis, i.e., thermally assisted hydrolysis and methylation gas chromatography/mass spectrometry (THM-GC/MS) with trimethylsulfonium hydroxide (TMSH) as the methylating reagent. The THM-GC/MS technique has been used to measure resin acid components (Simon et al. 2001; Watts and de la Rie 2002; Scalarone et al. 2003) phenolic acids (Zhang 1993; Zapf and Stan 1999; Yokoi et al. 2003) and lignins (Vane 2003; Klingberg et al. 2005) in complex biomaterials.

Materials and Methods

Plant Material and O_3 Treatment Needles used in this investigation were from treated branches of grafted clones of two genotypes used in a clonal orchard experiment described by Charland et al. (1994). The experiment involved ozone fumigation of 2-yr-old branches of 6-yr-old grafted white pine saplings (ramets) originating from Sudbury, Ontario (ozone-tolerant clone) and the Acadia Forest Experimental Station, New Brunswick (ozone-sensitive clone). Each ramet carried one replicate of all the branch treatments and received carbon-filtered air (10–15 ppb), 80 ppb, or 200 ppb ozone through separate branch chambers on randomly selected 2-yr-old branches. For this investigation, branches were fumigated daily for 40 d with O_3 [treatment profiles mimicking naturally occurring event shape maximizing at 10–15 (filtered air), 80 or 200 ppb] using a branch chamber fumigation system as outlined in Charland et al. 1995.

Experimental Design The experiment consisted of randomly distributed clonal plots of white pine within a uniform old nursery. Each plot contained at least six evenly spaced ramets. Three randomly chosen ramets (replicates) were shaded (57% shade) with shade cloth enclosures, whereas the other three were left in the open. Two-year-old branches on each ramet were randomly selected for treatment fumigations with the use of the Canadian Forest Service In-canopy branch chambers fumigation system. The system is further described in Charland et al. (1994, 1995).

Needle Samples Needles (40 from each plant) were removed from branches of each ozone treatment. Sampling was done at the same time of day to avoid diurnal fluctuations. Needles were dried at 60°C overnight, then ground to a fine powder before being stored in an airtight vial at −20°C.

Thermally-Assisted Hydrolysis Methylation Analysis THM-GC/MS analyses were carried out with TMSH as the preferred methylating reagent for resin acids as suggested in previous studies (Pastorova et al. 1997; Madonna et al. 2001). A small amount of ground needles (~250 μg) was placed into a pyrolysis sample cup, and 5 μl of TMSH (25% in methanol, Sigma-Aldrich) was added. The methanol was evaporated under a stream of N_2 before the sample cup was placed in standby position of the furnace pyrolyzer. The sample/TMSH mixture was then subjected to thermochemolysis by dropping the pyrolysis cup into the 400°C pyrolysis zone. Identification of the methylated phenolic and resin acid constituents of pine needles was based on retention times and mass spectral data of standard methylated phenolic standards and from compiled mass spectra of methylated diterpenoid resin acids (Zinkel and Magee 1987). Relative concentrations of phenolic and resin acids are reported as TIC (total ion chromatogram) peak area of individual compounds per μg of sample analyzed. THM-GC/MS was carried out by using a vertical microfurnace pyrolyzer (Py; Frontier Lab) interfaced to an HP5890 GC equipped with an HP 5971A MS as described in a previous paper (Estevez and Helleur 2005). A Frontier Lab UA-5 capillary column (25 m×0.25 mm i.d.; 0.25 μm thickness) was used. The instrumental settings were as follows: Py furnace, 400°C; Py interface and GC injector, 280°C; column pressure, 10 psi He; split flow, 20 ml/min; GC oven program, 100 to 230°C at 20°C/min, to 240°C at 1.5°C/min, to 300°C at 20°C/min; MS interface temperature 280°C, 70 eV, full mass scan mode from 40–550 amu.

Statistical Analysis Mean and standard error of the methylated phenolic acid compounds 1 and 3 were calculated from resulting chromatographic mean peak area per μg of sample. Statistical analysis of mean peak areas of these compounds found in needles exposed to ozone treatment, and shade/sun treatment for each clone-type were conducted by using the ANOVA statistical software PROC GLM in SAS (v.8.02).

Results

Metabolites Identified by THM- GC/MS The resulting total ion chromatograms of the TMSH thermochemolysis products of fumigated needles of ozone-tolerant and ozone-sensitive white pine are shown in Fig. 1. Five common phenolic and four distinctive diterpenoid resin acids were identified as their permethylated derivatives (Table 1). The original structures of these phenolic and resin acids are shown in Fig. 2. Although the original purpose of this study was to examine concentration changes in phenolic acids caused by O_3 fumigation, chromatographic profile differences of the resin acids between ozone-tolerant and ozone-sensitive clones were also observed (Fig. 1) showing a phenotypic variation within the two white pine clones. This distinctive resin acid profile may become useful in distinguishing tree populations that can be differentially adapted to air pollutants.

All major resin acids identified are labdane-type resin acids common in pine needles (Fig. 2). Anticopalic acid has been reported to be the principal resin acid in *P. strobus* needles (Zinkel and Spalding 1972) and was the most abundant resin acid in both pine clones. Three other resin acids, 3-oxoanticopalic, 3β-hydroxyanticopalic, and 3,4-cyclo-anticopalic acids, were identified as the other major resin acids in needles of the ozone-

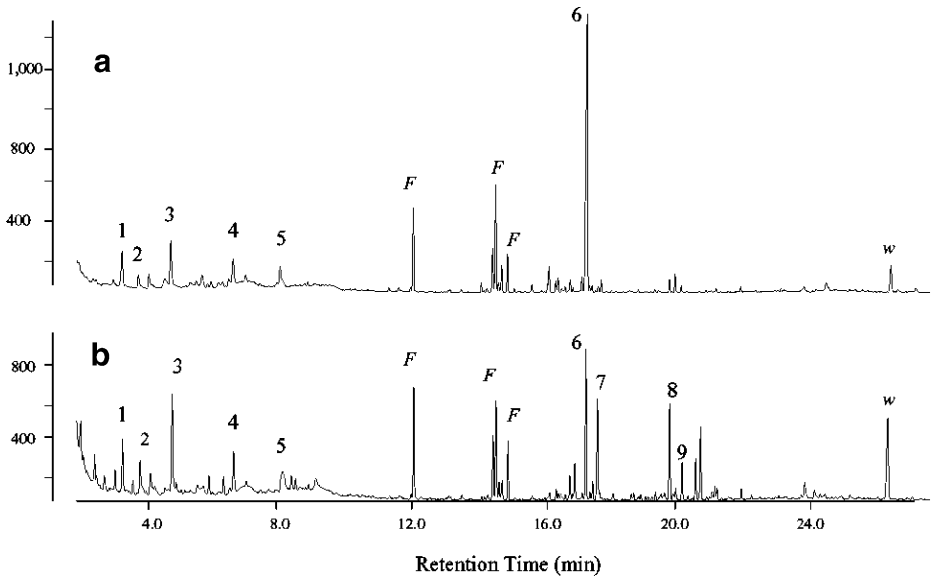


Fig. 1 Total ion chromatogram of the thermally assisted methylation products of **a** needles of ozone-tolerant *P. strobus* (200 ppb O_3 treatment.) and **b** needles of ozone-sensitive *P. strobus* (200 ppb O_3 treatment); the identity of the numbered peaks are listed in Table 1 and their nonmethylated structures shown in Fig. 2; *F*, fatty acid methyl esters, *w*, 10-nonacosanol

Table 1 List of permethylated phenolic and resin acids identified in *P. strobus* needles using thermally assisted methylation GC/MS

Peak[a]	RT (min)	Identity	Molecular Weight
1	3.45	3-Methoxybenzoic acid, methyl ester	166
2	3.82	4-Methoxybenzoic acid, methyl ester	166
3	4.58	3,4-Dimethoxybenzoic acid, methyl ester	196
4	8.74	2-Propenoic acid, 3-(4-methoxyphenyl) methyl ester	192
5	8.32	2-Propenoic acid, 3-(3,4-dimethoxyphenyl) methyl ester	222
6	17.51	Anticopalic acid, methyl ester	318
7	17.86	Cycloanticopalic acid, methyl ester	316
8	19.52	3-Oxoanticopalic acid, methyl ester	332
9	20.14	3β- Hydroxylanticopalic acid, methyl ester	334

[a] Peak numbers are those labeled in Fig. 1

sensitive pine. Conifer needles are also rich in epicuticular waxes. The major wax component of *P. strobus* as identified in Fig. 1 is the long chain alcohol, 10-nonacosanol.

Phenolic Acid Levels among Different Treatments and White Pine Populations Only 1-yr-old needles showed statistically meaningful variation in phenolic composition. 3-Hydroxybenzoic and 3,4-dihydroxybenzoic acids showed significant effects ($P<0.05$) with ozone exposure (Table 2). The combined results for both clones are graphically shown in Fig. 3. The sun/shade experiment had no statistically significant differences. These ozone-responsive phenolic compounds decrease in concentration with increased ozone dosage, by as much as 75% of the control level in the case of 200 ppb ozone (Fig. 3). At the 80 ppb ozone treatment, the ozone-tolerant pine (Sudbury clone) showed a more noticeable decrease in both phenolics, whereas both clones showed a substantial decrease at high exposure (200 ppb), as illustrated in Fig. 4.

Resin Acids and Ozone Treatment There was no noticeable change in resin acid concentrations in the needles of either the ozone-tolerant or ozone-sensitive white pines with O_3 fumigation. A new but unidentifiable chromatographic peak (RT=20.83 min, Fig. 1) was observed in needles of ozone-sensitive white pine fumigated at high (200 ppb) ozone levels. Its mass spectrum resembled a diterpene resin acid with a molecular weight of 332 amu. It is possible that this compound is an oxidized form of anticopalic acid formed as the result of high levels of oxidant. Further experimentation and mass spectral characterization is required to identify this product.

Discussion

This study involved the chemical profiling of phenolic and diterpene resin acids in needles of two (ozone-tolerant and ozone-sensitive) white pine clones fumigated with different ozone levels [12–15 (control), 80 and 200 ppb] for 40 d under field conditions. The metabolites of needles were successfully measured by using a relatively new approach to "whole sample" chemical profiling, that being TMSH-GC/MS. Short-term O_3 fumigation affects the levels of two phenolic acids, i.e., 3-hydroxybenzoic and 3,4-dihydroxybenzoic acids, in that both showed a substantial decrease in concentration with increased ozone exposure; as much as a 75% decrease in relative concentration (Figs. 3 and 4).

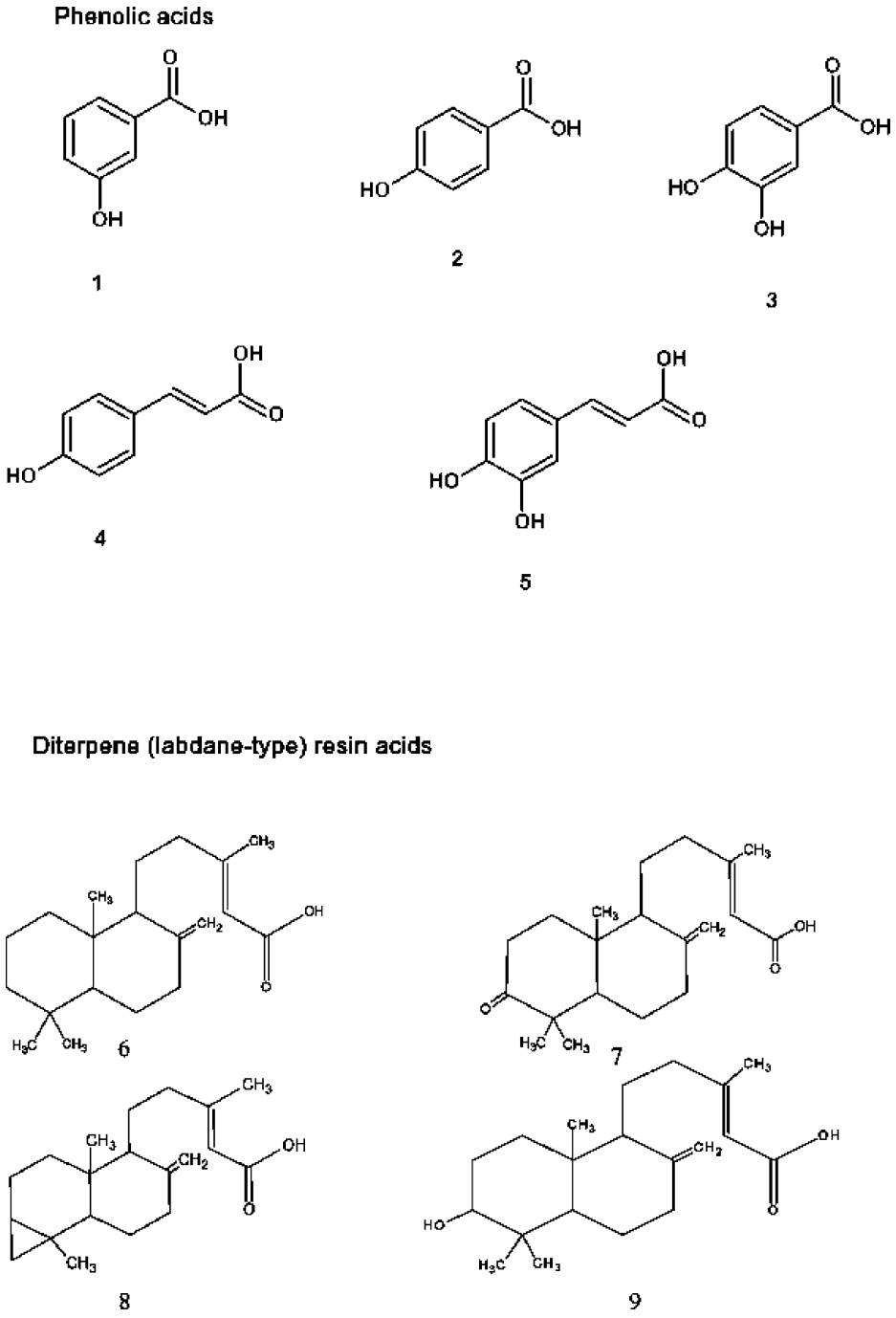


Fig. 2 Structures of phenolic acids and diterpene resin acids identified by THM-GC/MS in white pine needles. Compound *1* (3-hydroxybenzoic acid) and *3* (3,4-dihydroxybenzoic acid) were used as phenolic indicators of ozone exposure. The diterpene resin acids are anticopalic (*6*), 3-oxoanticopalic (*7*), 3,4-cycloanticopalic (*8*), and 3β-hydroxyanticopalic acids (*9*)

Table 2 ANOVA table for mean peak area/μg of sample of compounds 3-hydroxybenzoic acid and 3,4-dihydroxbenzoic

	df	Mean Square	*F* Value	*P*>*F*
3-hydroxybenzoic acid				
Ozone Treatment	2	10033467.11	19.10	<0.001
Clone	1	5028218.45	9.57	0.005
Sun/Shade	1	618075.12	1.18	0.288
Rep	2	18016.85	0.03	0.966
3,4-dihydroxybenzoic acid				
Ozone Treatment	2	4181318.00	19.58	<0.001
Clone	1	2237615.46	10.48	0.003
Sun/Shade	1	421018.81	1.97	0.172
Rep	2	46563.59	0.22	0.806

These two phenolic acid-THM products likely originate from the photosynthetic shikimate pathway that produces significant amounts of aromatic-containing secondary metabolites (i.e., flavonoids, tannins, and lignin). It is believed that ozone either inhibits the shikimate synthetic pathway, thus decreasing the amounts of 3-hydroxybenzoic acid and 3,4-hydroxybenzoic acid intermediates, or alters the pathway in preference of the synthesis of other secondary metabolite intermediates (Zhang 1993). A previous study (Katoh et al. 1989) determined that the shikimate pathway was affected in cedar needles by exposure of the plant to air pollution, including the oxidant SO_2.

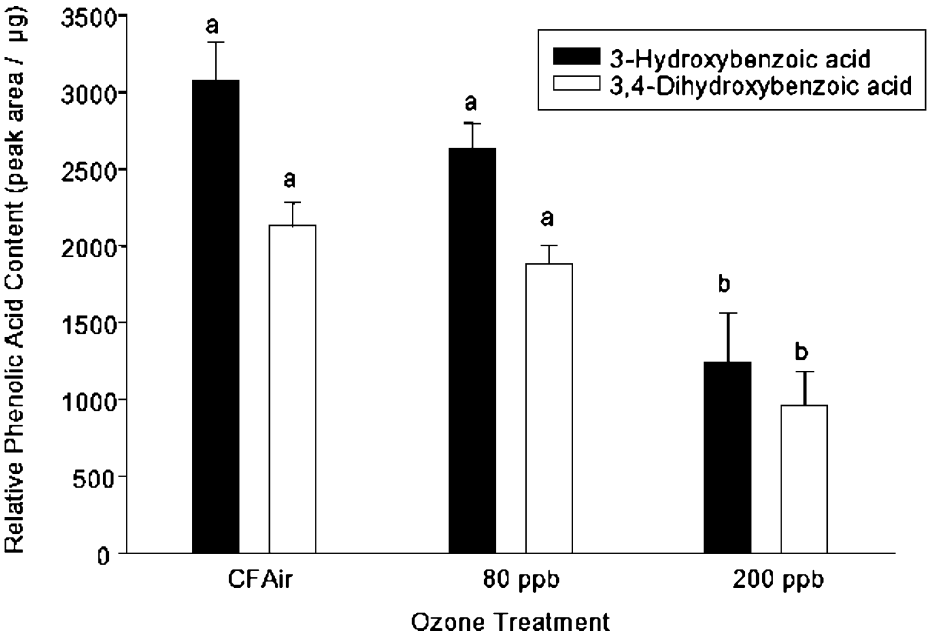


Fig. 3 Effect of ozone treatment on levels of phenolic acid compounds, 3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid of all clone samples. Data values used for each sample were mean of triplicate chemical analyses. Mean values shown are from three replicate exposures ±1 SE. *CFAir*, carbon-filtered air. *Letters* indicate Duncan's separation of means of compound ($P<0.001$)

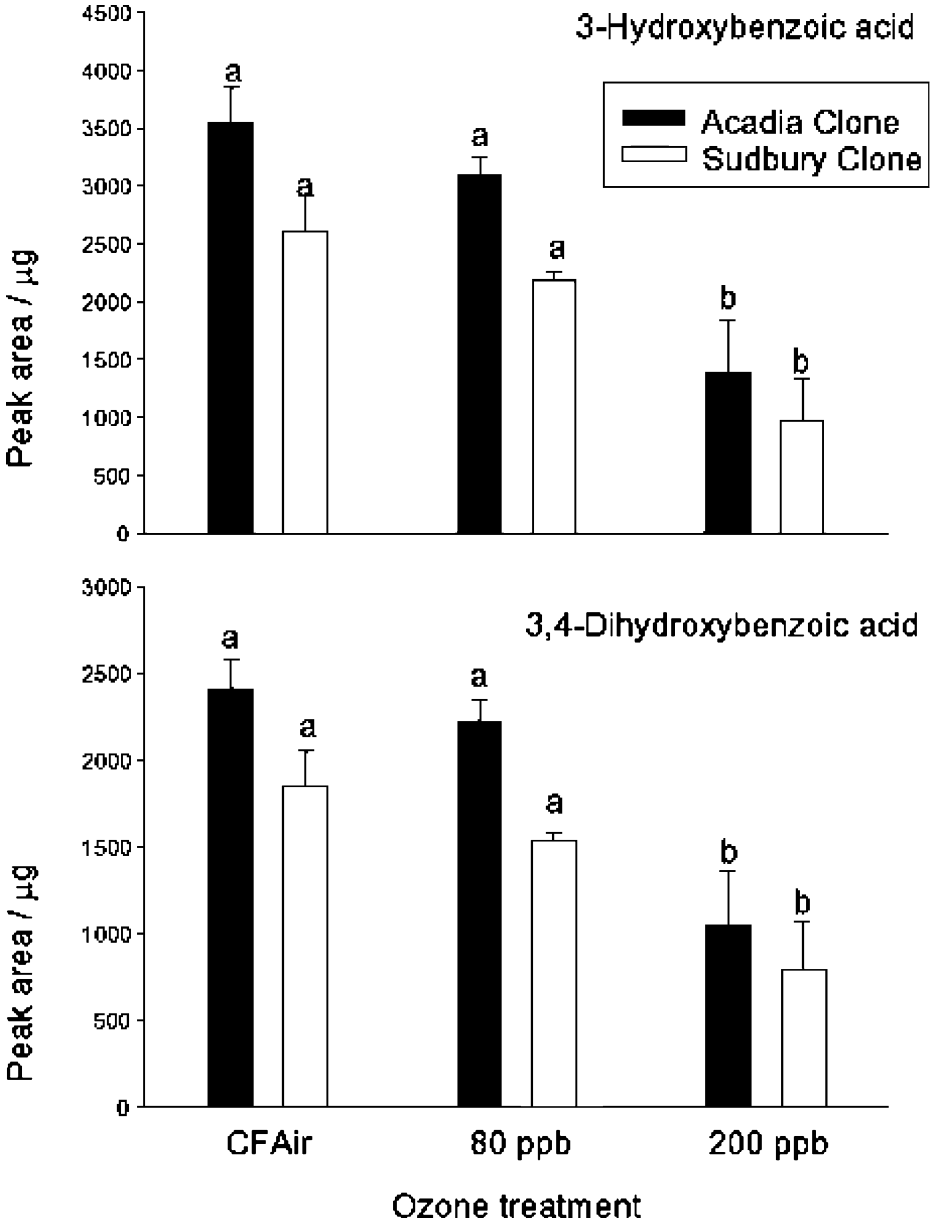


Fig. 4 Variation in relative content of 3-hydroxylbenzoic acid and 3,4-dihydroxybenzoic acid in needles of ozone-sensitive (Acadia clone) and ozone-tolerant (Sudbury clone) *P. strobus* fumigated with ozone. Data values used for each sample were the mean of triplicate chemical analyses. Mean values shown are from three replicate exposures ±1 SE. *CFAir*, carbon-filtered air. *Letters* indicate Duncan's separation of means by clone ($P<0.001$)

Our study has shown the presence of two ozone-sensitive chemical markers. Monitoring of these two phenolics may play a future role in biomonitoring of ozone levels and its impact on forest productivity. Our experimental approach of using rapid THM-GC/MS analysis is compatible with rapid chemical profiling of biomaterials as it involves little

sample preparation, uses no extraction solvents, and has the advantage of utilizing the whole sample, which allows for other biomarkers to be profiled simultaneously (i.e., fatty acids, terpenes, waxes, phenols, etc.).

Compositional differences in the major diterpene resin acids were observed between the needles of ozone-tolerant and ozone-sensitive pine populations. The four major resin acids, anticopalic, 3-oxoanticopalic, 3β-hydroxyanticopalic, and 3,4-cycloanticopalic acids, were present in the ozone-sensitive pine, whereas only anticopalic acid was present in the ozone-tolerant pine (Fig. 1). This phenotypic variation in resin acids within these white pines may be useful in distinguishing populations that may be differentially adapted to air pollutants.

THM-GC/MS data from the white pine needles fumigated at high ozone exposure (200 ppb) indicate the presence of an oxidized diterpene resin acid product. Future research will be undertaken to identify this and possibly other O_3 oxidation products as additional chemical markers in ozone-exposed trees.

Acknowledgment The authors thank Frontier Laboratories (Japan) for allowing the use of the vertical microfurnace pyrolyzer. Funding from NSERC and Memorial University is acknowledged.

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Introduction

The minute pirate bugs, family Anthocoridae (Hemiptera: Heteroptera), includes about 500 species worldwide, many of which are important predators in agroecosystems (Lattin 1999). As their name implies, minute pirate bugs are small (1.5–4.5 mm), and most are general predators of small soft-bodied insects and mites (Henry 1988). At least in North America, anthocorids in the genus *Orius* are significant predators across the widest range of crops of all the predacious heteropterans (Yeargan 1998), particularly in crops where they are able to supplement their carnivorous diet with pollen (Coll and Ridgway 1995; Coll and Guershon 2002). Some *Orius* spp. are also commercially available for augmentative biocontrol in greenhouse horticulture (Glenister 1998).

Minute pirate bugs often exploit cues associated with prey to direct their foraging (Lattin 1999). The most clear-cut and extreme example of this phenomenon is for *Elatophilus* spp., which use the sex pheromones of their prey, *Matsucoccus* spp. (Homoptera: Margarodidae), as kairomones to find these prey (Mendel et al. 2004). Similarly, Teerling et al. (1993) demonstrated that *O. tristicolor* (White) intensified searching in the presence of the alarm pheromone (decyl and dodecyl acetates) of one of its preferred prey, the western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). For the insidious flower bug, *O. insidiosus* (Say), Beekman et al. (1991) found that successful biological control of thrips was host-plant-dependent. Volatiles emanating from plants being fed upon by potential prey are exploited by *Anthocoris* and *Orius* spp. as prey-finding kairomones (Dwumfour 1992; Drukker and Scutareanu 1993; Drukker et al. 1995; Venzon et al. 1999; VanLaerhoven et al. 2000). *Anthocoris nemoralis* (F.) has learned to associate the presence of the herbivore-induced plant volatile, methyl salicylate, with prey (Drukker et al. 2000). Subsequently, methyl salicylate and certain other herbivore-induced plant volatiles have been shown to attract a variety of beneficial insects in the field, including *O. tristicolor* (James 2003, 2005; James and Price 2004). In corn, *O. insidiosus* is attracted to chemicals emanating from corn silks, thereby steering them toward likely spots to find eggs of the corn earworm (silks) and the European corn borer (leaves near the corn ear; Reid and Lampman 1989; Reid 1991). Perhaps, the most unusual semiochemical relationship noted for anthocorids is attraction to and feeding on humans (Aldrich, personal observation; Henry 1988). This behavior is reminiscent of their close relatives, the bed bugs (Cimicidae; Schuh and Slater 1995), but *Orius* bites are intensely painful, whereas those of bed bugs are painless. Feeding on mammalian blood by *Orius* may be more prevalent than now appreciated (Krinsky et al. 1980), but the distance from which the bugs are attracted to mammalian body odors and the identity of the responsible chemicals are unknown.

Detailed investigations of an Oriental minute pirate bug, *Orius sauteri* (Poppius), have revealed another intraspecific level of chemical communication that modulates foraging (Nakashima et al. 2002) and mate-finding (Nakashima and Hirose 1999). Female *O. sauteri* avoided areas where the female itself had searched previously, but did not avoid areas searched by conspecific females (Nakashima et al. 2002). There was no evidence males used such a patch-marking system. However, *O. sauteri* males offered a choice between leaflets on which virgin or mated females had walked vs. leaflets not exposed to conspecific females spent significantly longer on female-treated leaflets (Nakashima and Hirose 1999). The patch-marking signal must be relatively volatile, as this mark faded after only an hour, whereas the female-produced trail sex pheromone is much more persistent and evidently does not attract males even from short distances.

Notwithstanding the discovery that *O. sauteri* males ultimately orient to the trail sex pheromone to find a mate (Nakashima and Hirose 1999), the possibility remains that *Orius* and

other anthocorids also produce pheromones attractive to conspecifics over distances beyond the confines of a single leaf or plant. Indeed, for some time, we have known that *O. insidiosus* exhibits a pronounced sexual dimorphism of the metathoracic scent gland secretion (Aldrich and Puapoomchareon 1996). Therefore, the research presented herein was conducted with *O. insidiosus* to: (1) determine if the female-specific volatiles are part of a long-range attractant pheromone; (2) test the attractiveness of certain herbivore- and pathogen-induced volatiles (Li et al. 2002); and (3) examine the importance of contact chemoreception in mate-finding.

Methods and Materials

Insects *O. insidiosus* used for natural product identifications and for olfactometer attraction bioassays were purchased from Bunting Biological Control (Colchester, UK), and Koppert Biological Systems (Romulus, MI, USA). Young virgin *O. insidiosus* adults were separated by sex under a dissecting microscope and maintained separately in clear 500-ml plastic containers with fine-mesh nylon screen lids in a growth chamber at 27±1°C, a 16:8-hr (L/D) photoperiod, and 60–65% relative humidity. Rearing containers were provisioned with Angoumois grain moth eggs [*Sitotroga cerealella* (Olivier); BioFac, Mathis, TX, USA] sprinkled onto the sticky side of 2×6.5 cm Post-it® notepad paper, distilled water in two cotton-plugged shell vials (1.4×6 cm), and green beans, *Phaseolus vulgaris* L., washed in a 5.25% sodium hypochlorite solution then rinsed with tap water. Egg sheets and fresh green beans were replaced four times per week.

O. insidiosus adults for arrestant bioassays originated from the Novartis I 500 Ori-line® strain (Oxnard, CA, USA). The colony was maintained as described above, except that prey consisted of eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Beneficial Insectary, Oak Run, CA, USA), and sterile wide-mouth quart mason jars covered with organdy cloth lids were used as rearing containers. Fresh beans and moth eggs were added three times a week. The used beans were removed and placed on elevated metal screens in mason jars lying on their side. Fresh beans and *Ephestia* eggs were added to these jars three times a week until the *O. insidiosus* eggs hatched, nymphs molted, and virgin adults of known age were segregated by sex by examination of the genitalia under a dissecting microscope.

Sample Collection Virgin 3- to 8-d-old male and female *O. insidiosus* adults were used for extractions. Whole-body extracts were prepared by immersing adults in CH_2Cl_2 (50 adults/150–200 µl CH_2Cl_2/sample) for 5 min, drying with anhydrous Na_2SO_4, and transferring the solvent extract to a clean cone-bottom vial with a syringe. Airborne extracts were prepared from batches of ca 250 males or females transferred to 15×1.5 cm i.d. glass columns with an aspirator after chilling in a refrigerator. Columns were provisioned with *Sitotroga* eggs on paper as above, and air was drawn over the bugs by house vacuum at 100 ml/min through a humidifier and an activated charcoal prefilter (30/40 mesh; Aldrich Chemical, Milwaukee, WI, USA) for 3 hr without the downstream sampling filter to eliminate possible contaminating volatiles. Then, a precision filter containing 2 mg of activated charcoal (Brechbühler AG, Switzerland) was connected downstream from the bugs and sampled continuously for periods up to 3 d in a room ~25°C with fluorescent lights on a 16:8 hr L/D cycle. Trapped volatiles were subsequently extracted from the filter with ~70 µl of CH_2Cl_2 and stored in a freezer until analysis. Metathoracic scent gland (MTG) extracts were prepared by dissecting the glands from CO_2-anesthetized insects under tap water (4 MTGs /25 µl CH_2Cl_2/sample), drying with anhydrous Na_2SO_4, and storing in a freezer until analysis. Tap water was used for dissections because the use of distilled water results in rapid swelling of tissue as a result of osmosis.

Chemical Analysis Samples were analyzed by gas chromatography (GC) on a DB-1 column (0.25-μm film; 30 m×0.25 mm i.d.; J&W Scientific, Folsom, CA, USA) or an SPB-1 fused silica capillary column (0.25-μm film; 20 m×0.25 mm i.d.; Supelco, Bellefonte, PA, USA) in a Varian 3500 GC with helium as a carrier gas (50 cm/sec), a temperature program from 50°C for 2 min to 235°C at 15°C/min hold 2 min, in the splitless mode with a flame ionization detector.

Electron impact mass spectral (EI-MS) data were obtained using a Hewlett Packard 5971 GC-MS instrument at 70 ev, with an HP-5 column (0.11-μm film; 25 m×0.2 mm i.d.), programmed as above. Some EI-MS data were obtained with a Finnigan 4500 GC-MS at 70 ev and a DB-1 column (0.25-μm film; 30 m×0.25 mm i.d.) with helium as the carrier gas (linear flow=40 cm/sec) programmed from 50°C for 2 min to 180°C at 5°C/min, then at 10°C/min to 260°C. Chemical ionization mass spectrometry (CI-MS), with CH_4 and NH_3 as the reagent gases, was performed with a Hitachi M-80B mass spectrometer at 70 ev, with an SPB-1 column (60 m×0.25 mm i.d.) programmed from 60 to 220°C at 3°C/min.

GC-FT-IR spectra for female whole-body extracts were obtained using a GC/IRD/MSD system (HP 5890/HP 5965B/HP 5970B) at IRD transfer lines of 250°C, flow cell 250°C, resolution 8 cm, with a TC-WAX column (60 m×0.32 mm i.d.; GL Sciences, Tokyo, Japan), programmed from 70 to 220°C at 3°C/min, with helium as the carrier gas (linear flow=40 cm/sec).

Chemical Standards The following were purchased commercially (chiral compounds were racemic): (*E*)-2,7-octadienol (98%; TCI America, Portland, OR, USA); (*E*)-2-octenal (97%), (*E*)-2-decenal (96%), (*E*)-2-hexenal (98%), (*Z*)-3-hexenal (92.5%), (*Z*)-3-hexenyl acetate (98%), (*E*)-β-farnesene (90%), and ethyl (*E*,*Z*)-2,4-decadienoate (95%; Bedoukian Research, Danbury, CT, USA); (*Z*)-3-hexen-1-ol (98%), indole (98%), (*E*)-nerolidol (90%), methyl jasmonate (95%), (*E*)-2-octenoic acid (85%), (*E*,*E*)-2,4-decadienal (85%), dodecane (99%), and tridecane (99%; Aldrich Chemical); linalool (95%; SCM Glidco Organics, Jacksonville, FL, USA); methyl salicylate (95%) (Fisher Scientific, Fair Lawn, NJ, USA).

(*E*,*Z*)-2,4-Decadienal was prepared by reduction of ethyl (*E*,*Z*)-2,4-decadienoate (10 g, 94.8%, Bedoukian) with diisobutylaluminium hydride (DIBAH) followed by Swern oxidation of the corresponding alcohol to the aldehyde as follows. A solution of DIBAH (50 ml, 1.0 M in hexane) was added dropwise for 40 min to a cool solution of ethyl (2*E*,4*Z*)-decadienoate at −70°C (acetone-dry ice bath) under nitrogen. After 2 hr, under the same conditions as above, an additional 50 ml of DIBAH (1.0 M in hexane) was added dropwise to the reaction mixture to react completely with the remaining ester. Then, the mixture was gradually warmed to room temperature, poured into an ice-cold aqueous solution of NH_4Cl, and extracted with ether. The ether layer was successively washed with saturated $NaHCO_3$ (aq), brine, dried with $MgSO_4$, and concentrated. The crude product was filtered through silica gel (15 g) and eluted with 350 ml of hexane/EtOAc (85:15). The filtrate was concentrated to give a quantitative yield of (*E*,*Z*)-2,4-decadienol with 89.8% geometrical purity as checked by GC. This alcohol was subjected to Swern oxidation without further purification. Dry dimethyl sulfoxide (8.6 ml) was added dropwise to an acetone-dry ice cooled solution of oxalyl chloride (ClCOCOCl, 5 ml) in dry CH_2Cl_2 (100 ml) under nitrogen. After stirring for 5 min, a solution of (*E*,*Z*)-2,4-decadienol (7.7 g) in dry CH_2Cl_2 (10 ml) was added dropwise to the Swern reagent. The reaction mixture was stirred at −50–60°C for 20 min, then cooled to −70°C, and dry triethyl amine (35 ml) was gradually added to the reaction mixture. After stirring for 15 min, the reaction mixture was allowed to stand overnight in a refrigerator, poured into iced water, and extracted with pentane. The organic layer was successively washed with cool 1 N HCl (aq), water, saturated $NaHCO_3$, brine, dried with $MgSO_4$, and concentrated. The crude product was filtered through silica gel (10 g) and eluted with 200 ml of pentane. The filtrate was concentrated to give 6.9 g

(90.8%) of (*E*,*Z*)-2,4-decadienal. This was further purified by flash silica gel column chromatography (silica gel 250 g, eluted with hexane/EtOAc; 50:1) and distillation to give (*E*,*Z*)-2,4-decadienal (b.p. 73°C, 0.9 mmHg) with 83.9% geometrical purity.

(*E*)-2,7-Octadienal was prepared by conventional oxidation of (*E*)-2,7-octadienol with manganese dioxide (MnO_2). MnO_2 (13.9 g, Aldrich Chemical) was gradually added in one portion to a vigorously stirred solution of (*E*)-2,7-octadienol (5 g, 98% purity, TCI America) at room temperature. After 24 hr, the GC analysis showed 50% of starting material. Therefore, three additional amounts of 13.9 g of MnO_2 were added to the reaction mixture after 24, 45, and 66 hr to complete the reaction. Then, the reaction mixture was filtered through silica gel and eluted with CH_2Cl_2. The filtrate was concentrated and further purified by distillation (b.p. 82–84°C, 18 mmHg).

(*E*)-2,9-Decadienal and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were synthesized by previously described methods (Pattenden and Weedon 1968; Bestmann et al. 1989). (*E*)-2,7-Octadienoic acid was prepared by oxidation of the corresponding aldehyde. (*E*)-4-Oxo-2-octenal and (*E*)-4-oxo-2-decenal were obtained from a CH_2Cl_2 extract of first instar southern green stink bugs, *Nezara viridula* (L.) (Borges and Aldrich 1992).

Compounds were identified by comparison of their mass spectra to published mass spectra and/or the mass spectra of the authentic standards. Subsequently, all of the compounds tentatively identified by mass spectral data were cross-checked by comparison of the GC retention of the natural product to that of an authentic standard using the GC conditions described above and coinjection.

Y-tube Olfactometer Bioassay The responses of individual *O. insidiosus* adults to conspecific adults and extracts of adults were tested in a Y-tube olfactometer. The Y-tube was made from 8.0 mm i.d. Pyrex® glass with a base length of 6.75 cm and arms 6.5 cm in length at an angle of 45° to each other. Air to the olfactometer was supplied by a compressed air cylinder, regulated to 60 psi head pressure, passed through a charcoal filter (10 cm; 30/40 mesh) and water bubbler, then split into two streams, each of which entered a flow meter adjusted to provide 370 ml/min of air per arm. Silicone rubber tubing (Masterflex®, Cole-Parmer Instrument, Vernon Hills, IL, USA) was used to connect the humidifier and flow meters in line and to fasten glass tubes (3.7×0.6 cm o.d.) to each olfactometer arm. When live insects were used as a treatment, fine-mesh nylon screen was secured at both ends of the upstream glass tube to contain the insect; a maximum of five tests were performed with the same live insect in the source chamber. Drummond Microcaps® (5 μl; Thomas Scientific, Swedesboro, NJ, USA) filled with extracts or solvent and placed in the holding tubes served as dispensers for chemical treatments. Smoke was used initially to visualize the flow, verifying that there was no backflow from one arm into the other arm. Tests were conducted in a room (25±2°C) with fluorescent overhead lighting, with the Y-tube laid horizontally on a light table covered with white paper to provide diffuse bottom lighting.

Only virgin adults were used as source insects and test subjects. Single source insects were introduced into the upstream holding tube by placing the open end of the tube over a bug and allowing the insect to walk into the tube, then securing the screen on the other end and fastening the tube onto one arm of the olfactometer; the control holding tube on the other arm was empty. Test insects were introduced into the base of the Y-tube as described above, which seemed to minimize disorientation of the bugs. The test started once the Y-tube was laid down horizontally. Test insects were given 10 min to respond. A positive response was scored when an individual entered an arm of the olfactometer and remained for at least 30 sec, at which point, the test was stopped. Insects that failed to choose either arm of the Y-tube were excluded from analyses. Each age class (3- to 4-, 5- to 6-, and 7- to 8-d-old insects) was

tested about 20 times for a given treatment, and treatments were interchanged between arms after ten trials. Test insects were used for only one test. After each series of tests, the Y-tube and holding chambers were washed in hot detergent, rinsed with acetone, and dried in an oven for 30 min.

Preparation of Lures Lures for field tests were prepared by applying CH_2Cl_2 solutions of the various treatments to gray rubber septa (9 mm, Soxhlet extracted overnight with CH_2Cl_2; West Pharmaceutical Services, Kearney, NE, USA) and allowing the solvent to evaporate in a fume hood. Treatments tested in the field are listed in Table 1. Treatment A was a solvent only control; treatment B consisted of the major female-specific compound alone, (*E*)-2,7-octadienal; treatment C contained (*E*)-2,7-octadienal plus the major alkenal common to both sexes, (*E*)-2-octenal; treatment D added (*E*)-2,9-decadienal and (*E*)-2-decenal to components in blend C; treatment E consisted of compounds reportedly induced by caterpillar feeding on corn seedlings (Turlings et al. 1991), a favorite host of *O. insidiosus* (Yeargan 1998); and treatment F contained methyl salicylate and methyl jasmonate, herbivore- and pathogen-induced plant volatiles (Li et al. 2002) previously found to be attractive to various beneficial insects (James 2005). For treatments B–D, the most abundant compound in each blend was arbitrarily assigned a concentration of 0.5 mg active ingredient per 100 μl of CH_2Cl_2, and concentrations of the other compounds in these blends were determined based on the ratio of their chromatogram area counts to that of the most abundant compound in the blend. Components in blend E were combined according to their percentages as found by Turlings et al. (1991). Methyl salicylate and methyl jasmonate in treatment F were tested at a 10 mg each/100 μl CH_2Cl_2.

Field Trapping Tests of potential attractants were conducted during the 1999 growing season at the University of Maryland Agricultural Research Farm and the US Department

Table 1 Chemical treatments tested in field traps

Treatment	Blend	mg/100 μl CH_2Cl_2	Mean±SE[a]
A	CH_2Cl_2 (control)	–	0.8±0.1c
B	(*E*)-2,7-octadienal	0.5	1.2±0.1b
C	(*E*)-2,7-octadienal	0.15	3.7±0.4a
	(*E*)-2-octenal	0.5	
D	(*E*)-2,7-octadienal	0.15	1.4±0.1b
	(*E*)-2-octenal	0.5	
	(*E*)-2,9-decadienal	0.02	
	(*E*)-2-decenal	0.05	
E	(*Z*)-3-hexenal	1.96	0.4±0.1d
	(*E*)-2-hexenal	0.008	
	(*Z*)-3-hexenol	0.4	
	(*Z*)-3-hexenyl acetate	0.6	
	linalool	0.012	
	(3*E*)-4,8-dimethyl-1,3,7-nonatriene	0.012	
	indole	0.008	
	(*E*)-β-farnesene	0.4	
	(*E*)-nerolidol	0.008	
F	Methyl salicylate	10	0.4±0.1d
	methyl jasmonate	10	

[a] Means are the weekly number of males caught per trap per treatment. Means followed by a different letter are significantly different ($P<0.05$).

of Agriculture (USDA) Agricultural Research Center, Beltsville, MD, USA. Traps were deployed in plantings of *Bacillus thuringiensis* (*Bt*)-based transgenic sweet corn (Attribute GH0937- Rogers Brand, Novartis Seeds, Boise, ID, USA) in five plots ≥1 km apart as follows. Each plot had four plantings (April 27, May 10, June 1, and June 15) consisting of eight 30-m rows (~9,000 plants/ha; spacing ≈77 cm), with drip irrigation, and standard fertilizer and herbicide applications. Six delta sticky traps (Agrisense, Fresno, CA, USA) randomly baited with a lure for one of the treatments hung inside the trap were placed 9 m apart on stakes in each planting from 1 June–15 September. Traps were elevated each week to a height equal to the top of the corn plants in that particular planting until they were 1 m off the ground, at which height, they remained for the rest of the experiment. Traps were rebaited, and the sticky trap liners (Trécé, Salinas, CA, USA) were replaced weekly. Used trap liners were brought to the laboratory and examined under a microscope to count the number of *O. insidiosus* male and female adults captured.

Arrestant Bioassays Individual fifth-instar nymphs were placed in Petri dishes (5.7-cm diameter) with a small piece of chlorinated-rinsed green bean and a 1 × 1 cm piece of sticky notepad paper with *Ephestia* eggs. Newly eclosed male and female adults were maintained in separate mason jars with food, and mated individuals used for experiments were allowed to mate in rearing jars for 24 hr before use. Adults of the desired age and mating status were transferred individually to 28-ml plastic cups (Jet Plastica, Hatfield, PA, USA) covered with a cardboard lid and a 4.25-cm filter paper in the bottom and held in a growth chamber under conditions described above. After 24 hr, the exposed filter paper was removed and immediately placed 2 cm apart from an untreated control filter paper in a covered 10-cm glass Petri dish on a countertop in a room approximately 25°C with fluorescent lights. Test insects (i.e., responders, the same age as the treatment insect) were placed in the middle of the Petri dish using an aspirator and were observed for 10 min. The time spent by the responder on the filter papers (treatment vs. control) and elapsed time spent on each filter paper per visit were recorded. Bioassays were conducted between 10:00 A.M. and 3:00 P.M.

The first experiment tested the response of virgin *Orius* males to filter paper exposed to conspecific virgin females ranging in age from 1–6 d old. In each of these choice tests, the male was the same age as the female used in the treatment. Tests were conducted using 1-, 2-, 4-, and 6-d-old females, and each test was repeated 12 times with different filter papers and male adults.

The second experiment tested the response of virgin *Orius* females to filter paper exposed to conspecific virgin females or males ranging in age from 1–4 d old. In each of these choice tests, the female was the same age as the *Orius* adults used in the treatment, and each test was repeated six times on two different days with different filter paper treatments and *Orius* test females.

A third set of experiments was conducted in a similar manner as the first two experiments except that 2- to 4-d-old virgin male *Orius* responders were also tested against a treatment prepared by exposure of filter paper to 2- to 4-d-old mated *Orius* females. Paired choice tests were repeated 12 times over three different days using 2- to 4-d-old *Orius* test males as responders to filter papers exposed to 2- to 4-d-old conspecific virgin or mated females, 2- to 4-d-old virgin females vs. untreated filter paper, 2- to 4-d-old mated females vs. control paper, and 2- to 4-d-old virgin males vs. the control.

Statistical Analyses Y-tube olfactometer bioassay data were analyzed using the Wilcoxon signed-ranks test (Gravetter and Wallnau 1992). Field trapping data and the responses of *O. insidiosus* males to the arrestant produced by female *O. insidiosus* at various ages, were analyzed using analysis of variance PROC MIXED (SAS Institute 1996). If these field or

arrestant data did not meet the underlying assumption of homogeneity of variances, subsets of data with common variances were partitioned before analysis. When F values were significant ($P<0.05$), treatment means were compared using the least significant difference ($P<0.05$) procedure. The experiments comparing male and female responses to filter paper exposed to various *O. insidiosus* sources were analyzed separately using PROC TTEST (SAS Institute 1996). Tests for equal variance were conducted on the data set for each test day, and the appropriate test for either equal or unequal variances was used. Mean comparisons with $P<0.05$ were significantly different.

Results

Identification of Volatile Chemicals Chemicals identified from extracts of *O. insidiosus* males and females are listed (Fig. 1). Extracts of both male and female *O. insidiosus* adults have sex-specific compounds, as well as compounds occurring in both sexes (Figs. 2, 3 and 4). *(E)*-2-Octenal (**3**) was the most prominent component produced by both males and females;

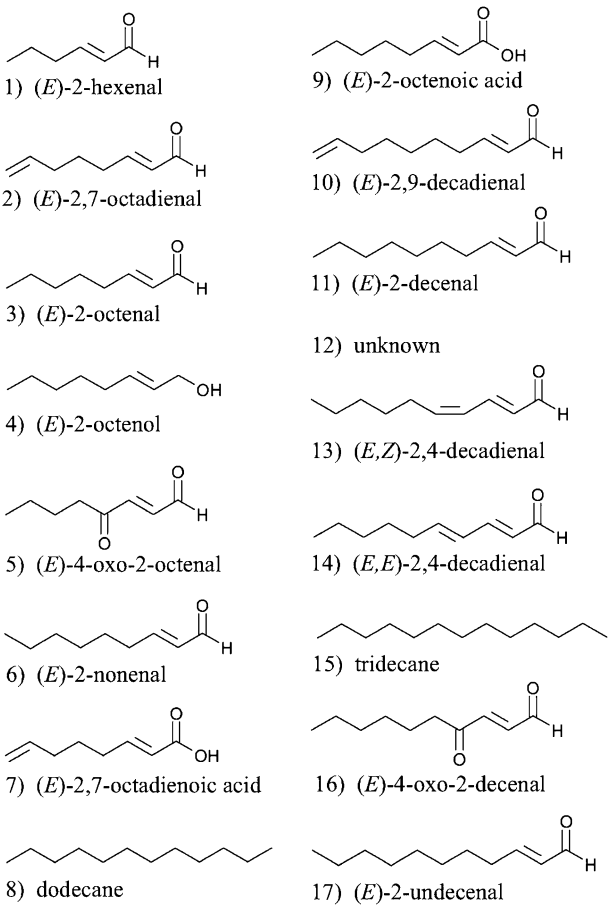


Fig. 1 Chemicals identified from extracts of *Orius insidiosus* adults

this compound was detected in whole-body extracts (Fig. 2), extracts of the metathoracic scent gland (Fig. 3), and aeration extracts (Fig. 4). Compound **2** eluted 0.11–0.15 min before (*E*)-2-octenal under the GC conditions employed and was observed only from females (all types of extracts; Figs. 2, 3 and 4). CI-MS indicated a molecular weight (MW) of 124, suggesting an octadienal structure, and FT-IR data exhibited an absorption peak at 3,085 cm^{-1}, characteristic of a terminal double bond (Leal et al. 1992). Compound **2** from natural extracts co-eluted with synthetic (*E*)-2,7-octadienal, thus, confirming the identity of this, the major female-specific volatile. Other female-specific compounds that were detected in whole-body and/or metathoracic gland extracts are compounds **7**, **10**, and **11** (Figs. 2 and 3), identified as (*E*)-2,7-octadienoic acid, (*E*)-2,9-decadienal and (*E*)-2-decenal, respectively. Compound **15**, identified as *n*-tridecane, was the major male-specific compound in all three types of extracts (Figs. 2, 3 and 4). The various other compounds not mentioned above and listed in Fig. 1 occurred at relatively low levels in either whole-body and/or metathoracic scent gland extracts (Figs. 2 and 3). Compound **a** observed in aeration extracts from both males and females (Fig. 4) has a MW of 126 based on CI-MS data, and may be the *(Z)*-isomer of octenal; compound **b** (MW 154) is an unidentified solvent impurity.

Y-tube Olfactometer Bioassay Live 3- to 4- and 5- to 6-d-old virgin *O. insidiosus* females were significantly attractive ($P<0.01$) to conspecific males of the same age, and whole-body extracts of 5- to 6- and 7- to 8-d-old virgin females significantly ($P<0.01$) attracted males (Fig. 5). On the contrary, virgin *O. insidiosus* males and extracts thereof were unattractive to conspecific females or, in the case of 5- to 6-d-old live males, were significantly ($P<0.01$) repellent to females (Fig. 6).

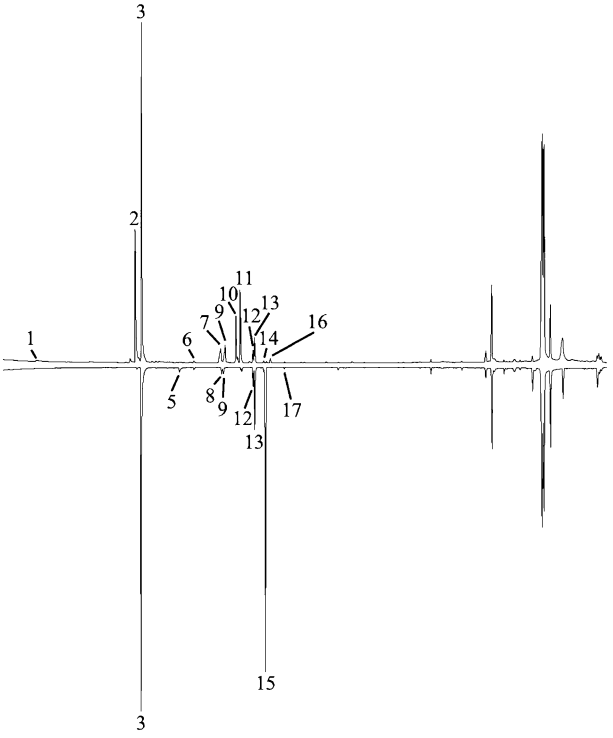


Fig. 2 Gas chromatograms and compounds identified from whole-body extracts of adult *Orius insidiosus* females (*top*) and males (*bottom*). Numbers correspond to structures in Fig. 1

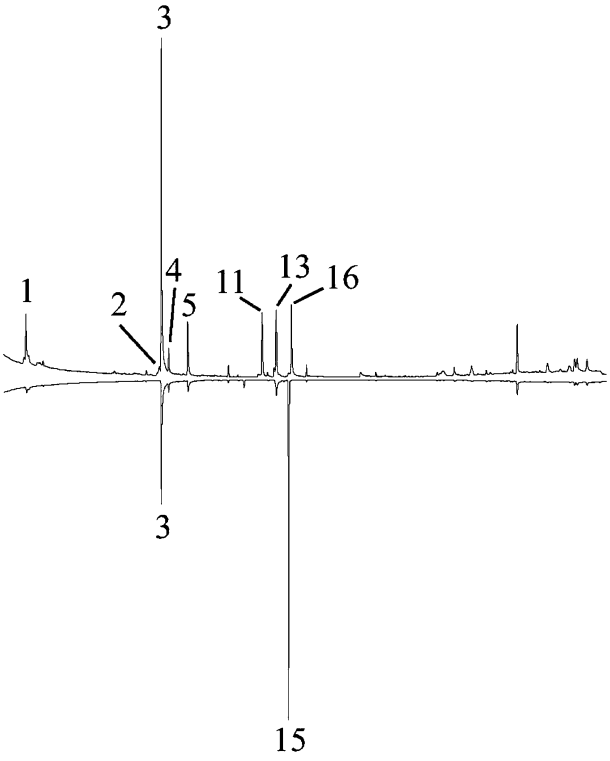


Fig. 3 Gas chromatograms and compounds identified from metathoracic scent gland extracts of adult *Orius insidiosus* females (*top*) and males (*bottom*). Numbers correspond to structures in Fig. 1

Field Trapping Significantly more males were caught in traps baited with (*E*)-2,7-octadienal and (*E*)-2-octenal than all other treatments (Table 1, C). The addition of other female-specific compounds (Table 1, D) resulted in a significant decrease in male attraction equivalent to that for (*E*)-2,7-octadienal alone (Table 1, B). Treatments E and F, designed to mimic volatiles induced by plant injury, attracted significantly fewer males than did the CH_2Cl_2-baited control traps.

Arrestant Bioassays The total time spent by virgin *O. insidiosus* males on filter paper exposed to virgin conspecific females was significantly greater than the time spent on untreated filter paper for all age classes tested (Table 2). The time spent per visit by males on filter paper exposed to females indicated that the arrestant effect was strongest when 1- or 2-d-old females were used to treat filter paper ($P<0.001$ in both cases) than when older females were used ($P<0.05$ for 4-d-old females and not significant for 6-d-old-females; Table 2).

The total time spent by virgin *O. insidiosus* females on filter paper exposed to virgin conspecific females was also significantly greater ($P<0.05$) than the time spent on untreated filter paper (Table 3). Conversely, females showed no preference for filter paper exposed to males vs. control filter paper, and in one data subset (test day 2; total time) avoided male-treated paper (Table 3).

Comparisons of the arrestant affect of virgin vs. mated *O. insidiosus* females toward conspecific males indicated that the mating status of females did not significantly alter the

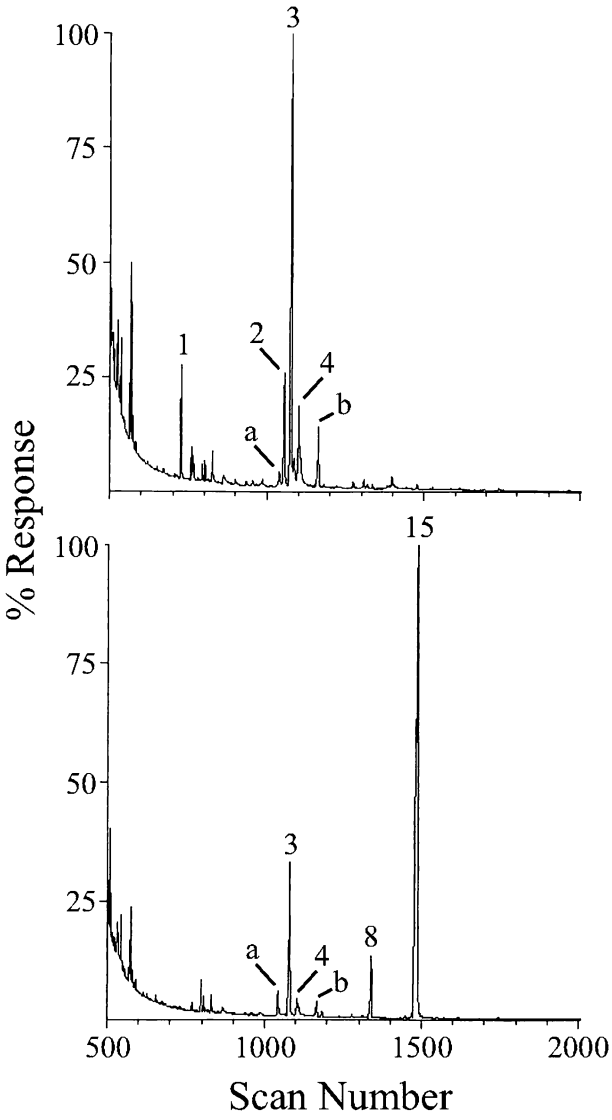


Fig. 4 Gas chromatograms and compounds identified from aeration extracts of adult *Orius insidiosus* females (*top*) and male (*bottom*) (*a* and *b* are an unidentified 126 molecular weight compound and a solvent impurity, respectively). Numbers correspond to structures in Fig. 1

positive response of males (Fig. 7). On two out of three test days, males were significantly arrested by filter paper exposed to virgin or mated females vs. untreated control filter paper (Fig. 7), an effect substantiated by data (not shown) recorded for time spent per visit by males responding to treated filter paper.

In general, before coming into contact with female-exposed filter paper, both males and females walked around the Petri dish with infrequent turns. Once they came in contact with the female-exposed filter paper, however, they usually stopped and briefly examined the paper, then appeared to walk faster and turn more frequently. If they walked off the filter paper, they usually returned quickly. Once an individual encountered the female-treated paper, it behaved in this manner for the remainder of the 10-min test.

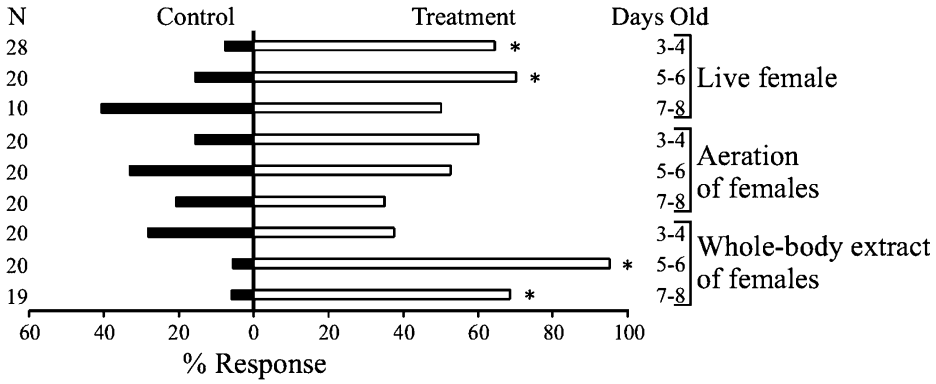


Fig. 5 Y-tube olfactometer bioassay results of virgin *Orius insidiosus* males of varying ages to conspecific females, aeration extracts of females, and whole-body extracts of females (*asterisk* denotes statistical significance at the 0.01 confidence level). Controls consisted of clean filter paper

Discussion

Orius insidiosus females produce a sex pheromone consisting of the female-specific compound, (*E*)-2,7-octadienal, and a compound emitted by both sexes, (*E*)-2-octenal. The *raison d'être* for the C_{10} female-specific compounds, (*E*)-2-decenal and (*E*)-2,9-decadienal, is unclear, as addition of these compounds to the octadienal/octenal blend actually reduced attraction of *O. insidiosus* males in the field. In any case, this system is another example of a heteropteran whose attractant pheromone consists of a sex-specific compound with a non-sex-specific compound, a situation encountered for various female plant bugs (Miridae) (Millar et al. 1997; Millar and Rice 1998; Zhang and Aldrich 2003) and male milkweed bugs (Lygaeidae: Lygaeinae) (Aldrich et al. 1997, 1999). Curiously, although the Anthocoridae and Lygaeidae are rather distantly related within the order (Schuh and Slater 1995) and opposite sexes produce the pheromones, the key pheromone component for milkweed bugs is also a terminally unsaturated octadienyl compound: (*E*)-2,7-octadienyl acetate (male-specific).

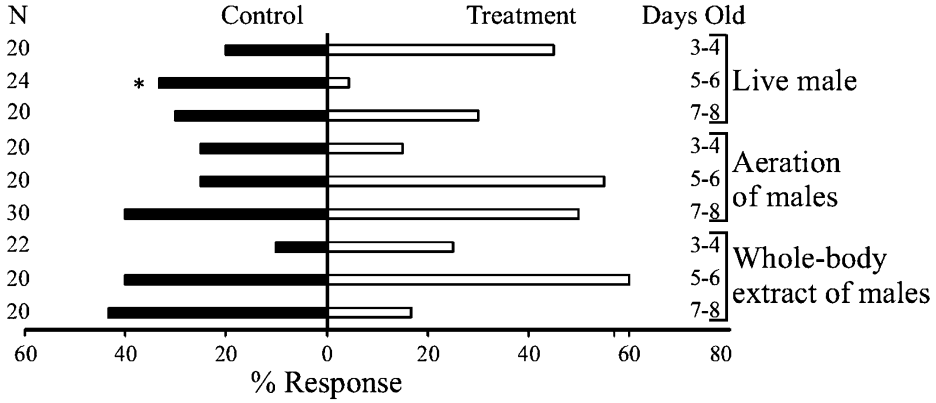


Fig. 6 Y-tube olfactometer bioassay results of virgin *Orius insidiosus* females of varying ages to conspecific males, aeration extracts of males, and whole-body extracts of males (*asterisk* denotes statistical significance at the 0.01 confidence level). Controls consisted of clean filter paper

Table 2 Total time spent and time spent per visit by virgin *Orius insidiosus* adult males[a] on filter paper exposed to conspecific virgin females of various ages for 24 hr vs. clean filter paper

Total Time (X±SE)					Time/Visit (X±s.e.)		
Age of treatment Female (d)	*N*	Treatment (sec)	Control (sec)	*P*	Treatment (sec)	Control (sec)	*P*
1	12	193±26	11±4.7	<0.001	45±9.8	5.3±1.3	<0.001
2	12	172±26	16±4.7	<0.001	30±4.8	6.3±1.3	<0.001
4	12	95±11	25±4.7	<0.001	39±9.8	10.0±4.8	<0.05
6	12	106±27	30±11.0	<0.05	21±4.8	15.0±4.8	NS

[a] Test males were the same age as the treatment female.

Clearly, the *Orius* pheromone components come from the metathoracic scent gland, as do those of mirids and lygaeines, so this is yet another case of a heteropteran insect producing its sex pheromone in the gland whose primary, probably ancestral, function is defensive (Aldrich 1988).

Our tests of synthetic lures that mimic induced corn volatiles and herbivore- or pathogen-induced plant volatiles (treatments E and F, respectively; Table 1) did not result in any demonstrable attraction of *O. insidiosus* adults. On the contrary, significantly fewer *Orius* adults were caught in traps baited with these lures than in control traps. In particular, we expected to observe a positive effect for the methyl salicylate/methyl jasmonate lure because the western Nearctic pirate bug species, *O. tristicolor*, was attracted to methyl salicylate in the field (James and Price 2004). Possibly, this discrepancy is caused by differential responsiveness between *O. tristicolor* and *O. insidiosus*. A more likely reason may be dose-related: James and Price (2004) used sachets that continuously released up to 60 mg of methyl salicylate per day, whereas our traps were baited weekly with lures containing a total of 10 mg of methyl salicylate.

Research on the trail (Nakashima and Hirose 1999) and patch-marking (Nakashima et al. 2002) pheromones of *O. sauteri* support the contention that female dispersion is primarily influenced by food resources, whereas male dispersion is guided more by the need to find mates (Nakashima and Hirose 2003). As *Orius* spp. search for prey by walking on plants, it is not surprising that females finally advertise their presence to males via non-volatile chemicals deposited on the substrate (Nakashima and Hirose 1999). For *O. insidiosus*, we

Table 3 Total time spent and time spent per visit by virgin *Orius insidiosus* adult females[a] on filter paper exposed to conspecific virgin females or males for 24 hr vs. clean filter paper

Total Time (sec; X±SE)					Time/Visit (sec; X±SE)		
Test Day[b]	*N*	Female[c]	Control	*P*	Female[c]	Control	*P*
1	6	223±79	12±11	0.04	87.0±39.0	6.4±5.6	0.09
2	6	222±56	68±27	0.03	77.0±20.0	25.0±7.7	0.04
		Male[c]	Control		Male[c]	Control	
1	6	73±48	125±61	0.52	22.0±15.0	85.0±64.0	0.38
2	6	34±19	119±32	0.04	8.7±2.6	34.0±11.0	0.06

[a] Test females were the same age as the treatment insect.

[b] Due to heterogeneity of variances, these data were analyzed as subsets performed on two different days.

[c] Test insects were virgin 1- to 4-d-old adults.

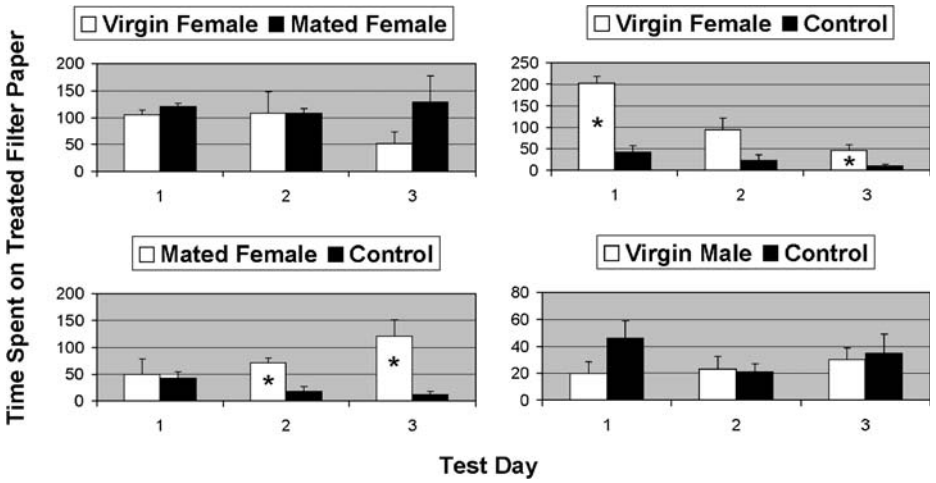


Fig. 7 Arrestment of virgin *Orius insidiosus* males given a choice of filter papers exposed to conspecific virgin females, mated females, virgin males, or untreated controls (*asterisk* denotes statistical significance at the 0.05 confidence level)

have likewise found that virgin males are strongly arrested and stimulated to search in areas previously exposed to conspecific virgin or mated females. Additionally, we showed that virgin *O. insidiosus* females were retained and stimulated to search the area of filter paper exposed to conspecific females. The behavior triggered in *O. insidiosus* males by substrate-borne chemicals from conspecific females is obviously adaptive for a male seeking a mate; however, it seems unlikely that a female orients to chemicals from a conspecific female to improve her chances of mating. In fact, the response of *O. insidiosus* females to chemicals from conspecific females is contrary to the bulk of evidence on marking pheromones in phytophagous and parasitic insects where females avoid resources marked by conspecific, congeneric, or even cross-ordinal females (Doumbia et al. 1998; Hemptinne et al. 2001; Nufio and Papaj 2001; Ruzicka 2001a, b; Laubertie et al. 2006). Perhaps prey or pollen sought by foraging *Orius* females are not so limiting as to result in competition requiring avoidance of conspecific foragers. On the contrary, the prior presence of conspecific females may be a cue (sensu Nufio and Papaj 2001) that potential prey are in the vicinity.

The semiochemical milieu of minute pirate bugs, particularly *Orius* spp., provides a glimpse of the reliance that these tiny insects have on chemical communication. Foraging is chemically influenced by plant synomones (Reid and Lampman 1989; Beekman et al. 1991; Reid 1991), perhaps even pollen odors (Dobson and Bergstrom 2000; yet to be investigated for *Orius* spp.), host allomones (e.g., Teerling et al. 1993), and incidental cues (kairomones) from potential prey (Nakashima and Hirose 2003). Evidence presented herein suggests that a foraging female is able to sense chemically the past presence of conspecific females, information that she may factor into her ongoing foraging strategy (Nakashima et al. 2002). *Orius* females can even privately communicate to themselves that they have recently explored a certain territory (Nakashima et al. 2002). Besides prey-related semiochemicals, there are mate-seeking signals such as trail or arrestant pheromones (data presented herein and Nakashima and Hirose 1999) and longer range volatile sex pheromones, as we have shown for *O. insidiosus*. Our discovery of a sex pheromone for *O. insidiosus* is tempered by the reality that trap catches per week were very low (approximately four males). This reality may be indicative that the volatile sex pheromone is a weak signal among those employed for

mate-finding or that there remain technical deficiencies in the research such as improper dosages/release rates of the pheromone or missing active components.

The complexity of semiochemical relationships in the Anthocoridae tends to reinforce emerging information on the semiochemical relationships of other Heteroptera. For example, among the phytophagous terrestrial bugs whose members undoubtedly produce long-range attractant pheromones (McBrien and Millar 1999), there is convincing evidence that egg parasitoids detect female-specific host cues by intercepting both volatile pheromonal cues (Leal et al. 1995) and non-volatile cues (Colazza et al. 1999; Borges et al. 2003). In the case of chemical footprints exploited by stink bug egg parasitoids (Colazza et al. 1999; Borges et al. 2003), it is certain that the female stink bugs are not leaving these chemical traces to help parasitoids find their eggs. Pentatomid adults are evidently tuned into each other through non-volatile chemical signals in ways that at present can only be surmised. Similarly, tachinid fly parasitoids exploit the volatile pheromones to find potential heteropteran hosts (Aldrich 1995b; Aldrich et al. 2006), but the bugs are not totally helpless in this regard because coevolution has endowed at least some species with non-volatile cuticular compounds that blunt their recognition by tachinid parasitoids (Aldrich 1995a). Concurrent with the chemical channels of communication, many land bugs communicate via substrate vibrations at close range (e.g., Çokl et al. 2001). In fact, male bugs may modulate pheromone emission in response to vibratory signals from conspecifics (Miklas et al. 2003). Finally, some heteropterans apparently utilize the pheromones of competitors as kairomones to find suitable host plants (Endo et al. 2006). This type of interspecific cross-attraction may be more common in the Heteroptera than currently appreciated (Slaymaker and Tugwell 1984; Tada et al. 2001; Aldrich et al. 2007).

From a practical standpoint, the fact that only *O. insidiosus* males are attracted to the octadienal/octenal pheromone blend limits the utility of this pheromone for enhancing biological control. A more fruitful target for a useful artificial semiochemical with which to enhance the biocontrol potential of the insidious flower bug and other *Orius* spp. may be the non-volatile female-specific compounds shown here to arrest females as well as males.

Acknowledgments We thank Sarah Ferguson and Tev Barros, CAIBL, for technical assistance and Dawn Harrison, BARC Diet and Human Performance Laboratory for assistance in obtaining mass spectral data.

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Introduction

Broad-spectrum and persistent insecticides have measurable ecotoxic impacts on the environment, as well as leaving undesirable residues in the human food chain. Alternative control tactics are urgently needed, and odorant-based tactics have already been demonstrated to offer valuable solutions for a range of pest species (El-Sayed 2007). Insects are highly responsive to natural and synthetic odorants used for mate and host location, and these innate responses have provided many opportunities for intervention and control with strategies based on volatile semiochemicals. Early use of odorants involved systems for monitoring, but in recent decades, they have increasingly been developed into direct control tactics (e.g., Minks and Cardé 1997; Suckling and Karg 2000).

In this paper, we propose a new multiple species interference system ("Ménage-à-Trois for Insects") in which we consider a system to control one or two species by disrupting mating (Fig. 1). We suggest that the placement of a sufficiently powerful odorant onto one species ("Species A") would act as a lure for individuals of a second species ("Species B") and interfere with mating of one or both species. Attractants could interfere with mating success of recipient first species by deliberately causing the second one to chase it, thereby interfering with its ability to find a mate (Ménage 1). We also hypothesized that the second species could fail to mate at the normal rate if individuals expended most of their effort in pursuing the lure instead of a conspecific mate (Ménage 2). To our knowledge, this concept has never been tested. This approach could lead to a new range of tactics for a wide range of target pests, including cross-order systems (e.g., Diptera-Lepidoptera) and could lead to a new sustainable pest management tactic with reduced insecticide use.

A number of criteria must be met for this novel concept to succeed with sympatric species: there must be a suitable way of loading the odorant onto the "lure" species; the odorant must not increase mortality or decrease fitness of treated individuals; the odorant must persist for long enough to attract the second species; the second species must be sufficiently attracted to actually reduce the level of mating.

Fruit flies (Diptera: Tephritidae) are significant economic pests of horticultural crops in many countries, and many different control tactics have been developed. These include male annihilation (mass-trapping of males), bait sprays, sanitation (removal of infested fruit from the field), biological control, and the sterile insect technique (Cunningham 1989). Semiochemical attractants have been used as part of fruit fly detection, eradication, or long-term pest management programs for decades (Beroza et al. 1961). These insects, therefore, offer a good system to test the concept, especially as they can be easily reared in large quantities.

We, therefore, selected oriental fruit fly *Bactrocera dorsalis* (Hendel) and melon fly *B. cucurbitae* (Coquillett) as in both of these species females are attracted to males for mating at dusk. The male attractants available in this system are methyl eugenol and cuelure, which are highly attractive to oriental fruit flies (Cunningham 1989) and melon flies (Keiser et al. 1973), respectively.

We focused on four main questions: (1) would the application of a semiochemical attractant for an individual cause increased mortality?; (2) could we apply sufficient attractant to one species that would induce significant attraction of the second species?; (3) would there be behavioral effects of this cross-species attractant application on the baited and the attracted flies?; and (4) what would be the effects of the application of the attractant on mating of the attracted species (Ménage 1 in Fig. 1) and on the baited one (Ménage 2)? These questions were addressed through a series of laboratory and field cage experiments to provide "proof of concept" for this new cross-species disruption concept.

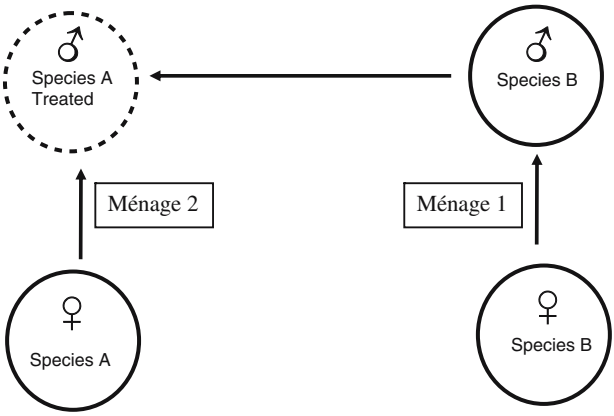


Fig. 1 Overview of components of the proposed "Ménage-à-Trois for Insects" system, where males of *Species A* are treated with an attractant for *Species B*, and the conspecific mating of each species is evaluated as an outcome

Materials and Methods

Insects Sterile laboratory-reared melon and oriental fruit fly pupae were obtained from the USDA-ARS Pacific Basin Agricultural Research Center's rearing facility in Honolulu, Hawaii. Pupae, shipped by air from Honolulu to Hilo, were subsequently held under natural photoperiod in 30×30×30-cm cubical screened cages at 26–28°C and 60–75% RH. Flies were immobilized after emergence, separated by sex in a cold room (4°C), placed in 11.5× 7.5-cm plastic containers (55 individuals/cage) with nylon mesh covers, and provided with water, sugar, and hydrolyzed protein. Laboratory colonies of oriental fruit fly with a sex-linked marker for white pupal color in females (McInnis et al. 2004) were used, where indicated, to reduce cost of sorting by sex. Virgin flies were typically 8–10 days old, unless otherwise indicated.

Toxicology The proof of concept for this approach requires that the odorants do not cause mortality to the treated insects (A). A dose response for methyl eugenol (4-allyl-1,2-dimethoxybenzene; 99.52%, Farmatech Int. North Bend, WA, USA) was obtained for topical application of 1 μl of acetone containing 0, 0.001, 0.003, 0.01, 0.03, 0.10, and 0.30 μl of methyl eugenol per fly onto the thorax of chilled (−10°C for <5 min) melon flies held on crushed ice for 2–3 min with a topical applicator (Hamilton PB600-1 Repeating Dispenser, Hamilton Company Reno, NV, USA). Fifteen mated flies of each sex (age) were treated per replicate (three replicates per dose, total of 45 flies of each sex per dose), which were then placed in cages containing water and protein at 22°C. Mortality was recorded at 1, 2, 3, 4, 7, 10, and 14 days after treatment. The identical procedure was used to determine the effect of cuelure [4-(4-acetoxyphenyl)-2-butanone; >96%, Interchem Technologies, Tijuana, Mexico] on oriental fruit flies.

Wind Tunnel Choice Tests Melon fly Treated with Methyl Eugenol. Virgin male melon flies (11–12 days old) were chilled (as above) and topically dosed with 1 μl of 10% methyl eugenol in acetone (100 ng) on the thorax and allowed to recover for several minutes. Treated and untreated (control) males were then rechilled and tethered by the thorax with cotton thread attached by cyanoacrylate superglue (Instabond Super Glue, Accrabond, Olive Branch, MS, USA). The other end of the tether (c. 5 cm length) was tied to the top of a 2.5-cm entomology pin, which was then stuck through the center of a 15-cm-diameter filter paper (no. 2, Whatman UK, Maidstone, UK) into a rubber bung (2.5 cm high). This

enabled the tethered flies to move freely around the flat circular arena above a clean paper background platform (15×21 cm) at 30 cm elevation above the ground. Quality assurance tests of treated and tethered flies showed the capacity for sustained flight in the laboratory. The two arenas (treated and control flies) were placed at the upwind end and in the middle of a flight tunnel (1×1×2 m length, Jang and Light 1991) operated at 0.1 m/s wind speed, 275 lux. The left and right placement of the treatment and control pair was alternated between replicates. There was a distance of 5 cm between the lower paper platforms and the same distance between the edges of the circular arenas.

Each replicate used newly treated tethered flies and circular arenas, as well as 25 virgin flies (10–14 days old) released simultaneously 1.3 m downwind from the lure flies. There were a total of 16 replicates performed, each one lasting 15 min. The occurrence of the following behaviors were recorded for Species B (oriental fruit flies): landing (present on the upper surface of the filter paper), approach (<1 cm) to the tethered Species A, physical contact (bumping or licking Species A), substrate feeding (i.e., on regurgitate), and the total number of flies involved in arena behaviors during the assay. For species A (melon flies), avoidance (walking) or escape (tethered flight or jumping) behaviors were recorded.

Oriental Fruit Flies Treated with Cuelure. This wind tunnel choice experiment was repeated but using 100 ng of cuelure applied to oriental fruit flies (Species A) in the presence of 25 melon flies (Species B) (16 replicates).

Field Cage Olfactometer The attractiveness of treated Species A to a known population size of Species B was assessed. Jackson traps (Epsky et al. 1996) for oriental fruit flies were baited with either a single treated melon fly or filter paper (100 or 300 ng of methyl eugenol) on a rotating olfactometer in a screened 3×3×2.5-m field cage (Jang et al. 1997). One hundred virgin male oriental fruit flies were then introduced, and traps were checked after 24 h. The same protocol was used, releasing 100 virgin melon flies into the cage when traps were baited with either single treated oriental fruit flies or filter paper (100 or 300 ng of cuelure). There were five replicates of each treatment for each compound.

Field Trapping with Treated Flies Jackson traps were used to determine the potential for field attraction of individual flies of the two species at a papaya orchard (Kapoho, HI; Latitude 19.51 N, Longitude 154.85 W). Virgin male melon flies were topically treated with 100 or 300 ng of methyl eugenol, and single flies were placed onto the sticky base. Filter papers (4.25 cm, Schleicher & Schuell Inc., Keene, NH, USA) treated with the same loadings and folded into a raised triangle were used as a positive control. Fifteen replicates

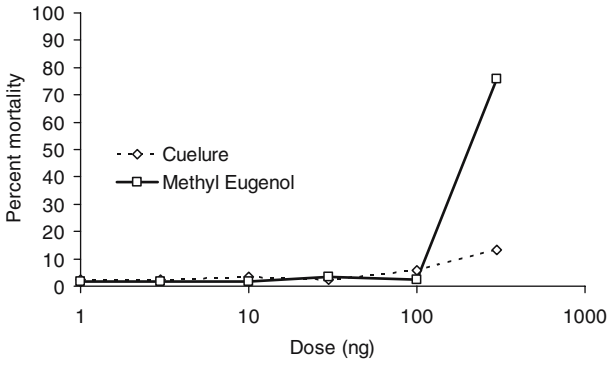


Fig. 2 Dose responses of oriental fruit flies and melon flies to topically applied cuelure and methyl eugenol 1 week after treatment

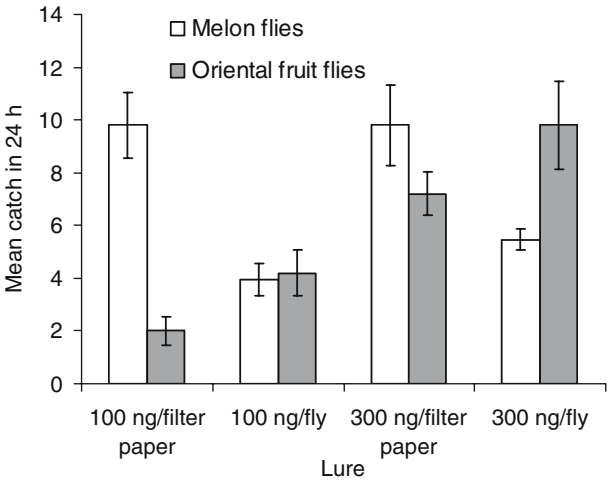


Fig. 3 The number of wild oriental fruit fly and melon fly males attracted to heterospecific males or filter papers treated with 100 ng of methyl eugenol or cuelure, respectively, in Jackson traps over 24 h at a papaya orchard in Hawaii

of each treatment were prepared and checked for catch after 24 h. Again, the same protocol was used with male oriental fruit flies treated with 100 or 300 ng of cuelure along with filter paper controls with these loadings. Catch per trap (15 replicates) was also recorded after 24 h.

Trapping in Large Field Cage The longevity of the attractive effect of 100 ng of methyl eugenol on living melon flies to oriental fruit flies was determined by simultaneously testing individuals treated 6, 3, 2, 1, and 0 days before the experiment. Two replicates of fly-baited Jackson traps (along with a blank control) were hung on plants in a large screen cage (4.2×10.6×1.7 m, of C32A mesh, Synthetic Industries, Gainesville, GA, USA). Traps were hung in blocked lines with trap positions randomly assigned within each replicate (line). Approximately 1,000 sterile virgin male melon flies were then released into the cage. Traps were checked after 24 h. This test was repeated on three occasions, providing six replicates in total.

Mating Incidence in Small Cages The mating success of sexually mature virgin flies of both Species A and B (Ménage 1 and Ménage 2 in Fig. 1) was assessed in six treatments for each lure. For Species A, the impact on fitness was determined by comparing mating of treated males and untreated females with or without the presence of a male of Species B, with control pairs, to determine the effect of treatment on Species A mating. For Species B,

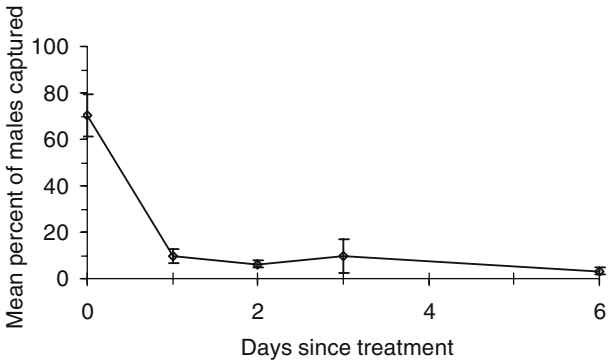


Fig. 4 The number of sterile oriental fruit fly males captured in Jackson traps baited with melon fly males that had been treated with 100 ng of methyl eugenol at different times before the 24-h bioassay was run

Table 1 The means (+SEM) of attraction- and rejection-related behaviors in a 15-min wind tunnel choice test when oriental fruit flies (Species B) were exposed to melon flies treated with methyl eugenol or untreated controls (Species A)

Species – Parameter	Treatment (+SEM)		Control (+SEM)		*P*
B – landing	19.33	(3.79)	0.47	(0.33)	0.001
B – approach (<1 cm)	13.67	(1.93)	0.07	(0.06)	0.001
B – contact	139.47	(31.71)	0.00	(0.00)	0.001
B – substrate feeding	2.07	(0.86)	0.00	(0.00)	0.005
B – no. of flies participating	3.36	(0.50)	0.07	(0.07)	0.001
A – avoidance (walking)	6.13	(1.05)	0.00	(0.00)	0.001
A – escape (flight)	4.00	(0.99)	0.00	(0.00)	0.002

the effect of male displacement through chasing treated males of Species A on mating incidence of Species B was similarly assessed.

For methyl eugenol, the ménage treatments (1 and 2) were: (a) a methyl eugenol-treated melon fly male with one male and female oriental fruit fly; (b) a control melon fly male with one male and one female oriental fruit fly; (c) just one male and one female oriental fruit fly; (d) one methyl eugenol-treated male and one female melon fly with one male oriental fruit fly; (e) a control male and one female melon fly with one male oriental fruit fly; and (f) a control male and a female melon fly. The first three treatments tested the potential of Ménage 2, whereas the second three treatments tested Ménage 1.

Melon fly males were treated topically with 0.1 μl of methyl eugenol in 1 μl of acetone (100 ng) or 1 μl acetone alone (controls). Forty pairs of each of the six combinations were caged in aluminum mesh cylinders (15 cm diameter, 15 cm tall) covered with plastic wrap over each end held with rubber bands and placed into a covered outdoor field cage under natural light 4 h before visual assessment of mating incidence (i.e., pairs in copula) at 8:00 P.M., 1 h after the start of scotophase (7:00 P.M). Forty pairs of each of the six combinations were caged in aluminum mesh cylinders and placed into a covered outdoor screen field cage for 24 h before visual assessment of mating.

The equivalent protocol of six treatments was used for cuelure applied to oriental fruit fly males, again with 40 replicates.

Mating Incidence in Field Cages Eighty to one hundred virgin male oriental fruit flies were treated with 100 ng cuelure or acetone alone (control) and released together in the afternoon with the same number of virgin females, 80–100 pairs of virgin melon flies into a screen

Table 2 The means (+SEM) of attraction- and rejection-related behaviors in a 15-min wind tunnel choice test when melon flies (Species B) were exposed to oriental fruit flies treated with cuelure or untreated controls (Species A)

Species – Parameter	Treatment (+SEM)		Control (+SEM)		*P*
B – landing	11.00	(1.56)	1.87	(0.67)	0.001
B – approach (<1cm)	9.67	(1.19)	0.33	(0.18)	0.001
B – contact	148.20	(19.32)	0.00	(0.00)	0.001
B – substrate feeding	3.60	(0.67)	0.00	(0.00)	0.001
B – no. of flies participating	4.27	(0.54)	0.47	(0.13)	0.001
A – avoidance (walking)	6.07	(0.77)	0.27	(0.18)	0.001
A – escape (flight)	3.60	(0.93)	0.13	(0.13)	0.001

See also Supplementary Material.

Table 3 Mating incidence of virgin oriental fruit flies and melon flies in small cages with and without treatment of melon fly males with methyl eugenol

Ménage-à-Trois Treatment 1			Total Pairs of Oriental Fruit Flies	Percent of Oriental Fruit Flies Mating
Methyl eugenol-treated melon fly male	Male Oriental fruit fly	Female Oriental fruit fly	40	82.5
Control melon fly male	Male Oriental fruit fly	Female Oriental fruit fly	40	90.0
–	Male Oriental fruit fly	Female Oriental fruit fly	40	72.5
Ménage-à-Trois Treatment 2			Total pairs of Melon flies	Percent of Melon fly mating
Methyl eugenol-treated melon fly male	Female melon fly	Male Oriental fruit fly	36	11.1
Control melon fly male	Female melon fly	Male Oriental fruit fly	40	95.0
Methyl eugenol-treated melon fly male	Female melon fly	–	38	23.7

cage (3×3×2.5 m), and mating pairs in copula collected 1 h after the onset of scotophase and assessed the next day. This experiment was replicated ×3. The experiment was also repeated with topical dosing of melon flies with 100 ng of methyl eugenol or acetone alone, and assessment of mating incidence was done in the same way.

Statistical Analyses Analysis of variance was performed on log-transformed trap counts to stabilize the variance (Zar 1984). Values were backtransformed by antilog for presentation in the original units. Chi-squared tests were used to analyze mating incidence and effect of odor toxicity by sex (Zar 1984). Paired *t*-tests were used to compare the incidence of

Table 4 Mating incidence of virgin oriental fruit flies and melon flies in small cages with and without treatment of oriental fruit fly males with cuelure

Ménage-à-Trois Treatment 1			Total Pairs of Melon Flies	Percent of Melon Flies Mating
Cuelure-treated Oriental fruit fly male	Male melon fly	Female melon fly	38	92.1
Control Oriental fruit fly male	Male melon fly	Female melon fly	40	100.0
–	Male melon fly	Female melon fly	40	92.5
Ménage-à-Trois Treatment 2			Total pairs Oriental fruit flies	Percent of Oriental fruit flies mating
Cuelure-treated Oriental fruit fly male	Female Oriental fruit fly	Male melon fly	40	80.0
Control Oriental fruit fly male	Female Oriental fruit fly	Male melon fly	40	90.0
Cuelure-treated Oriental fruit fly male	Female Oriental fruit fly	–	40	82.5

Table 5 Mating incidence of oriental fruit flies and melon flies in outdoor field cages after treatment of oriental fruit flies with 100 ng of cuelure or melon flies with 100 ng of methyl eugenol compared to controls

Treatment	Total Pairs Oriental Fruit Fly	Percent of Oriental Fruit Flies Mating	Total Pairs Melon Fly	Percent of Melon Flies Mating
Cuelure-treated Oriental fruit fly male	293	32.1	377	65.5
Control Oriental fruit fly male	300	56.3	282	66.0
Methyl eugenol-treated melon fly male	202	58.9	187	1.6
Control melon fly male	185	67.0	350	81.7

attraction- and rejection-related behaviors between treatment and controls in wind tunnel choice tests after log-transformation to stabilize the variance (Zar 1984).

Results

Toxicology There was no difference between sexes for either odorant at the highest dose (methyl eugenol χ^2=0.0, *df*=1, *P*=1.0; cuelure χ^2=3.086, *df*=1, *P*=0.079), and the data were, therefore, pooled for sex. The toxicity of methyl eugenol to melon flies was <5% at concentrations up to and including 100 ng per fly but dramatically increased in the 300 ng treatment (Fig. 2). The majority of the mortality occurred within 24 h (data not shown). A similar pattern was observed when oriental fruit flies were treated with cuelure (Fig. 2), although toxicity at 300 ng per fly was lower. Control mortality after a week was <2% for both species.

Field Cage Olfactometer Male oriental fruit flies dosed with 100 and 300 ng of cuelure were as attractive to male melon flies as filter papers loaded in the same way. There was no significant difference in catch between the four treatments ($F_{3,16}$=0.15, *P*=0.93; filter papers or flies at 100 and 300 ng loadings) with a backtransformed mean (+SEM) catch per trap per 24 h of melon flies to cuelure of 8.5+1.1 flies. Similar results were seen in the number of oriental fruit flies attracted to methyl eugenol at 100 and 300 ng loadings on filter papers or melon flies ($F_{3,16}$=0.49, *P*=0.7) with a backtransformed mean catch per trap per 24 h of 11.8+1.1.

Field Trapping Jackson traps baited with methyl eugenol-treated flies or filter paper caught wild oriental fruit flies. Similarly, traps baited with flies or filter paper loaded with cuelure attracted melon flies (Fig. 3). Significant differences were evident for both attractants between substrate and loading: oriental fruit fly to methyl eugenol ($F_{3,56}$=13.70, *P*<0.001); melon fly to cuelure ($F_{3,56}$=15.10, *P*<0.001). Filter papers were more attractive than flies loaded with cuelure, whereas higher catches were obtained with the higher loading (300 ng) of methyl eugenol.

Trapping in a Large Field Cage Recapture of oriental fruit flies was highly significantly for time since treatment (analysis of variance on angular transformed data; $F_{5,30}$=24.99, *P*< 0.001), and insects treated with methyl eugenol on the day of release yielded 70% of the total recaptures (Fig. 4), amounting to 15.1+2.3% of flies released. Catches in the blank controls were 1% (+0.5) of the total.

Wind Tunnel Choice Tests Male oriental fruit flies were attracted to methyl eugenol-treated male melon flies (Table 1), and in separate experiments, the male melon flies were attracted to cuelure-treated oriental fruit flies (Table 2, Supplementary Material). In both cases, the various parameters were different between treatment and control. Species B flies closely approached and interacted with treated males of the other species, which in turn showed avoidance behaviors. Aggressive chasing of treated flies by the other species appeared like conspecific aggressive behavior with a quick hit or head butt and jump backward.

Mating Incidence In small cages, there was no difference in mating incidence of oriental fruit flies (72.5–90%; Table 3) with or without methyl eugenol-treated melon flies present (χ^2=4.119, *df*=2, *P*=0.128). However, the mating of male melon flies was reduced by their treatment with methyl eugenol (Table 3). There was no effect of cuelure-treated oriental fruit fly males on melon fly mating nor did the treatment with cuelure affect their own mating (Table 4; χ^2=2.018, *df*=1, *P*=0.155).

However, in the larger outdoor field cages, there was a reduction in the mating incidence of oriental fruit flies treated with cuelure (Species A) compared to the untreated controls (Table 5; χ^2= 33.315, *df*=1, *P*<0.001). However, mating of melon flies (Species B) was unaffected by cuelure-treated oriental fruit flies (Table 5; ÷^{2}=2.519, *df*=1, *P*=0.112). Methyl eugenol treatment of melon fly males reduced their mating success compared to controls (χ^2=314.7, *df*=1, *P*<0.001; Species A), but there was no effect on the oriental fruit flies (Species B; χ^2=2.7, *df*=1, *P*=0.10).

Discussion

These experiments have demonstrated that 100 ng amounts of two types of attractants applied topically to laboratory-reared fruit flies were not toxic over the period of 1 week, thus meeting the first requirement of lack of toxicity for the model system. There was evidence that a topical dose of methyl eugenol of 300 ng was toxic to melon flies, and this higher dose was not examined further in behavioral and mating experiments, although it was included in trapping experiments for comparison with cuelure, which was not toxic at this dose.

The 100-ng dose of each lure attracted multiple individuals of the target species in an outdoor field cage and in the field. Results in the rotating olfactometer in a field cage with laboratory-reared flies were more consistent than results with wild flies, presumably because of the more controlled conditions. However, both types of experiments demonstrated potentially useful levels of attraction with living fruit fly lures loaded with 100 ng of attractants. The large field cage experiment indicated that male melon flies treated with 100 ng of methyl eugenol were effectively capable of attracting oriental fruit flies for 1 day. A greater longevity of effect would presumably be an advantage for such a system.

The 100-ng doses also induced an interesting range of close range behaviors that involved physical contact with the loaded flies both in the wind tunnel and in cages. In the most intense situation, where escape was partially prevented by tethering, physical contact between the chasing male occurred an average of about 10 times a minute for prolonged periods.

In one of the two attractant-fly systems examined, it is possible that a significant problem would arise whereby the treatment could increase mating incidence. For example, methyl eugenol is also a phago-stimulant capable of increasing the competitive mating fitness of males that have fed upon it (Shelly and Dewire 1994; Shelly et al. 2005). A similar, although short-lived effect, has been reported for cuelure (Shelly and Villalobos 1995); however, here, no significant difference in the mating incidence of melon flies was evident in the presence of cuelure-treated oriental fruit flies.

The final test of mating incidence in an outdoor field cage with cuelure applied to oriental fruit flies did show an impact on their overnight mating frequency. Methyl eugenol treatment reduced mating of melon flies even more dramatically. The fact that the cuelure or methyl eugenol treatments to Species A had no effect on mating of Species B flies in both cases appeared to be due to a behavioral switch from attraction to the para-pheromone lure earlier in the day to mating behavior over the crepuscular period. The incidence of lek formation in male flies on the ceiling of the field cage appeared to increase toward dusk, although it was not specifically recorded. In the field cage experiments with odorant-treated males, lower mating incidence at dusk was recorded, but accurate recording of male chasing of treated males was not practical, so such a behavioral explanation for the failure of this ménage-à-trois model would require further examination. A possibly analogous switch in behavior between mated and unmated flies to either male pheromone or host odor for oviposition has been reported by Jang (1995) for females of another tephritid, *Ceratitis capitata* (medfly). This behavioral limitation would appear to be a significant problem for these fruit fly models but would not necessarily apply in other cases. It is also possible that the topical treatment created repellency from treated males, effectively rendering them unattractive to females at close range. Alternatively, a sublethal impact with reduced male proclivity for mating may be responsible. Further experiments to examine this effect could be valuable, as this could eventually lead to a new tactic based on loading of wild male melon flies with methyl eugenol to reduce mating incidence.

A better system for the ménage-à-trois concept might offer a greater degree of behavioral synchrony between the elements of attraction and mating, which were separated by time in the two models tested here. For example, it might be more successful to interrupt mating in medfly (which mates in the morning) with the presentation of an attractant (such as cuelure or methyl eugenol) to other species. The potential impact of treatment of males of two species with reciprocal lures was not examined. It is possible that this would have a greater success rate against fruit flies than the single components alone.

Whereas it might be of interest to investigate the response of males of one species to male lures placed onto females of another species, this variation was also not examined here. This was in part because of the present lack of female lures for wild flies and the unlikelihood of a scenario of releasing laboratory-reared female flies, as they can damage fruit. However, the potential of this approach could be of interest to investigate in the future as it might facilitate the development of a tactic that uses hybrid sterility (a form of the sterile insect technique) in fruit flies (McInnis et al. 1999). Odorants attractive to males of another species that were placed on females of a closely related species could serve the function of bringing the pair into close proximity before the mating period. The potential for combination of the Sterile Insect Technique with the current approach is significant, given fruit flies as potential targets. However, if Species A were treated and innundatively released as part of a sterile insect program, impact on mating of the treated males (as seen here) could be detrimental to the control of Species A by SIT, which would require evaluation of the trade-off. Use of mass-reared medflies and other insects as a potential source of point source distribution of pheromone for mating disruption is under investigation.

Several of the main issues considerations for the proof of concept of this novel approach have been addressed here, although the impact on mating was limited or absent with these models. Another limitation to the approach is the requirement for both species in the system to be present at the same location. Although there are many locations where both of the fruit fly species examined here are present together, this requirement would limit the potential utility, unless the second species is released proactively, for example, as sterile

insects. Systems of large-scale odorant delivery would also need to be developed, and there are other practical issues that would need to be addressed. It may be possible to get this concept to work more effectively in a completely different model system. A much greater understanding of conditions under which the ménage-à-trois concept will or will not work is needed before practical application will be possible. This work offers novelty in presenting attractants on a moving target, in this case, another insect. Other applications for this concept remain to be developed.

Acknowledgments D. M. Suckling undertook this project with a Fellowship under the Organization for Economic Cooperation and Development Cooperative Research Programme: Biological Resource Management for Sustainable Agricultural Systems. Other support was provided by the US Department of Agriculture, Agriculture Research Service, HortResearch, and the New Zealand Foundation for Research Science and Technology (CO6X0301). Don McInnis provided invaluable insights into mating assessment in fruit flies and comments on the manuscript. Kellie Nagai helped with preparation of material for experiments.

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Keywords Pheromone biosynthesis · Unsaturated hydrocarbon pheromone · Polyenic hydrocarbons · Deuterium labeling · α-Oxidation · Deuterium-labeled pheromone precursor · Winter geometrid · Lepidoptera · Geometridae

Introduction

Erannis (recently renamed to *Agriopis*) *bajaria* Denis et Schiffermüller (Lepidoptera: Geometridae: Ennominae) is a south and central European winter-flying geometrid moth that is adapted to low temperatures. Females are wingless and climb up plant stems where they emit a pheromone blend to attract males in a similar way as described for *E. aurantiaria* and *E. defoliaria* (Tvermyr 1969). Known geometrid moth pheromones are usually long-chain polyunsaturated hydrocarbons and their oxygenated derivatives (Millar 2000). The pheromone blend of *E. bajaria* was reported to consist of (3*Z*,6*Z*,9*Z*)-3,6,9-octadecatriene (3*Z*,6*Z*,9*Z*-18:H) and (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (3*Z*,6*Z*,9*Z*-19:H) (Szöcs et al. 1996). Although 3*Z*,6*Z*,9*Z*-18:H attracts conspecific males as a single component, the addition of 3*Z*,6*Z*,9*Z*-19:H is strongly synergistic, and a 1:1 mixture of both compounds proved to be the optimal attractant for males in field bioassays (Szöcs et al., unpublished).

Polyene hydrocarbon pheromones are biosynthetically derived from linoleic acid or α-linolenic acid and usually contain an odd number of carbons in the chain with (6*Z*,9*Z*) or (3*Z*,6*Z*,9*Z*) arrangement of the double bonds. The precursor acids are chain elongated by two-carbon acetate units via malonate, when necessary, and the terminal carboxyl group is reductively removed by decarboxylation or decarbonylation (Jurenka 2004). This process produces pheromones with an odd number of carbon atoms, as shown in detail by applying labeled precursors to *Estigmene acrea* (Rule and Roelofs 1989). Less information is available about the biosynthesis of such pheromones with an even numbered chain, which occasionally occur as components of some pheromones, e.g., in *E. bajaria*. Direct reduction of the polar carboxyl moiety of polyunsaturated even numbered fatty acids, perhaps with corresponding aldehydes and alcohols as intermediates, was proposed by Millar (2000) and was recently described to be working in a bacterium (Park 2005). In contrast, we postulated that chain shortening might take place in two consecutive steps, removing one carbon in each step, producing hydrocarbons with one or two carbons less than the parent fatty acids. Therefore, experiments with labeled precursors were performed with *E. bajaria* to elucidate the pathway furnishing 3*Z*,6*Z*,9*Z*-18:H and 3*Z*,6*Z*,9*Z*-19:H.

Methods and Material

Preparation of Deuterium-Labeled Compounds Heating methyl linolenate (9*Z*,12*Z*,15*Z*-18:COOMe) with freshly prepared sodium methoxide in CH_3OD under reflux for 8 hr yielded the corresponding deuterated methyl ester [2,2-$^2H_2$]-9*Z*,12*Z*,15*Z*-18:COOMe in 95% yield. The deuterated methyl ester was reduced with lithium aluminum deuteride in refluxing diethyl ether to furnish pure [1,1,2,2-$^2H_4$]-9*Z*,12*Z*,15*Z*-18:OH in 89% yield. This alcohol was transformed to the corresponding iodide [1,1,2,2-$^2H_4$]-9*Z*,12*Z*,15*Z*-18:I under Mitsunobu conditions in 69% yield. Subsequently, the carbon chain was elongated by nucleophilic substitution with tetraethylammonium cyanide in 89% yield, followed by reduction of the cyanide with diisobutylaluminum hydride to form [2,2,3,3-$^2H_4$]-10*Z*,13*Z*,16*Z*-19:Al. This aldehyde was reduced to the corresponding alcohol which then was oxidized with pyridinium dichromate in dimethylformamide to furnish [2,2,3,3-$^2H_4$]-

10*Z*,13*Z*,16*Z*-19:COOH in 30% yield over two steps. Direct oxidation of the [$^2H_4$]-aldehyde resulted in a lower yield and an impure product that was difficult to isolate. [3,3,4,4-$^2H_4$]-11*Z*,14*Z*,17*Z*-20:COOH was obtained by similar transformations after converting [1,1,2,2-$^2H_4$]-9*Z*,12*Z*,15*Z*-18:I into [3,3,4,4-$^2H_4$]-(11*Z*,14*Z*,17*Z*-)-11,14,17-icosatrienenitrile by C_2-elongation with acetonitrile, followed by saponification of the nitrile in 61% overall yield (Taber and Kong 1997).

Insects Adults were collected at Nadap, Hungary and were kept under outdoor conditions at PPI HAS (Budapest) in 4-l glass jars equipped with sheets of filter paper as oviposition substrates. The following spring, eggs were placed on buds of blackthorn (*Prunus spinosa* L.) enclosed in sleeve cages. Before pupation, larvae were transferred to cages containing sterilized soil for pupation. Pupae were sexed, and emerging adults were collected daily.

Experiments with Labeled Precursors The deuterium-labeled compounds [1,1,2,2-$^2H_4$]-9*Z*,12*Z*,15*Z*-18:OH, [2,2,3,3-$^2H_4$]-10*Z*,13*Z*,16*Z*-19:COOH, and [3,3,4,4-$^2H_4$]-11*Z*,14*Z*,17*Z*-20:COOH were topically applied in DMSO solution (20 μg/μl; total amount applied, 1 to 2 μl/female) on the ovipositor tip. This procedure was performed 2 hr before the normal diel calling period. During the calling period, ovipositor tips (containing the pheromone gland) were dissected and extracted in hexane for 5 min. Extracts were stored at −65°C until analysis by gas chromatography–mass spectrometry (GC-MS). About 15 female equivalents were combined for one analysis.

Gas Chromatography–Mass Spectrometry GC/MS analyses were carried out on an HP 6890 Series GC connected to an HP 5973 mass selective detector (Hewlett-Packard) fitted with a BPX5 column (25 m, 0.22 mm i.d., 0.25-μm film, SGE). Mass spectra (70 eV) were recorded in both scan and multiple ion monitoring mode (MIM). The GC was programmed as follows: 2 min at 50°C, then to 320°C at 5°C/min, operating in splitless mode (60 sec valve time).

Results and Discussion

Several deuterium-labeled precursors were synthesized and topically applied to the pheromone glands of females of *E. bajaria*. Subsequently, extracts of the pheromone glands were analyzed by GC/MS. Because of the low pheromone titer, samples of about 15 female equivalents were pooled for analyses. After topical application of [2,2,3,3-$^2H_4$]-10*Z*,13*Z*,16*Z*-19:COOH, 17,17,18,18-$^2H_4$-3*Z*,6*Z*,9*Z*-18:H was detected in the extracts along with the unlabeled compound. Application of [3,3,4,4-$^2H_4$]-11*Z*,14*Z*,17*Z*-20:COOH also yielded the labeled triene, but in lesser amounts. In the latter case, all four ^2H atoms of the labeled acid were incorporated into 3*Z*,6*Z*,9*Z*-18:H. Incorporation of the deuterium label into the polyenes was checked by GC/MS in MIM scan mode. Mass spectra of 3,6,9-alkatrienes usually exhibit a molecular ion and a signal generated by loss of a 56-amu fragment that includes carbons 1 to 4 (Becker et al. 1983). In the case of [17,17,18,18-$^2H_4$]-3*Z*,6*Z*,9*Z*-18:H, the molecular ion (M^+) of the unlabeled compound (*m*/*z* 248) and its diagnostic fragment M^+-56 (*m*/*z* 192) should be shifted by 4 amu to *m*/*z* 252 and *m*/*z* 196, respectively. Table 1 shows the results obtained when MIM was performed using the ions *m*/*z* 192, *m*/*z* 196, *m*/*z* 248, and *m*/*z* 252. Mean incorporation rates of 5.7% in the case of [2,2,3,3-$^2H_4$]-10*Z*,13*Z*,16*Z*-19:COOH and 1.9% in the case of [3,3,4,4-$^2H_4$]-11*Z*,14*Z*,17*Z*-20:COOH were calculated from the ion intensities $I_{196}/(I_{192}+I_{196})$ and $I_{252}/(I_{252}+I_{248})$.

Table 1 Relative intensities of ions found in GC/MS multiple ion monitoring analysis at the retention time of 3*Z*,6*Z*,9*Z*-18:H[a]

	$I_{192}+I_{196}$	I_{196}	$I_{196}/(I_{192}+I_{196})$	$I_{248}+I_{252}$	I_{252}	$I_{252}/(I_{248}+I_{252})$
[D4]-10*Z*,13*Z*,16*Z*-19:COOH	106.5	6.5	0.061	105.6	5.6	0.053
[D4]-11*Z*,14*Z*,17*Z*-20:COOH	101.1	1.1	0.011	102.7	2.7	0.026
[D4]-9*Z*,12*Z*,15*Z*-18:OH	100.0	0	0	100.0	0	0

[a] The earlier elution of the deuterated isotopomer is accounted for by summing the intensities of the ions from the start until the end of both peaks. I_y Relative intensity of the ion of *m*/*z* y (%)

The analytical results proved that both 10*Z*,13*Z*,16*Z*-19:COOH and 11*Z*,14*Z*,17*Z*-20:COOH are biosynthetic precursors of 3*Z*,6*Z*,9*Z*-18:H. Furthermore, when topically applied to the gland, the alcohol [1,1,2,2-$^2H_4$]-9*Z*,12*Z*,15*Z*-18:OH did not provide labeled 3*Z*,6*Z*,9*Z*-18:H, which excludes the possibility that linolenic acid is directly reduced via the alcohol to 3*Z*,6*Z*,9*Z*-18:H, as proposed by Millar (2000). A biosynthetic pathway taking into account the results of our labeling experiments is summarized in Fig. 1. Linolenic acid, taken up from the diet and most likely conjugated to CoA, is chain-elongated in the conventional sequence with malonate to yield 11*Z*,14*Z*,17*Z*-20:CoA. α-Oxidation then results in loss of one carbon to give 10*Z*,13*Z*,16*Z*-19:CoA. This precursor is then converted into 3*Z*,6*Z*,9*Z*-18:H, probably via 10*Z*,13*Z*,16*Z*-19:Al (Reed et al. 1995; Jurenka 2004). In contrast, removal of C-1 of 11*Z*,14*Z*,17*Z*-20:CoA after the conventional process (Rule and Roelofs 1989) furnishes 3*Z*,6*Z*,9*Z*-19:H.

To the best of our knowledge, α-oxidation of unbranched fatty acids in animals has not been reported before, although α-oxidation of linolenic acid has been shown to occur in humans (Matsunaga et al. 1997). This mechanism must now be considered to be involved in pheromone biosynthesis in other species as well.

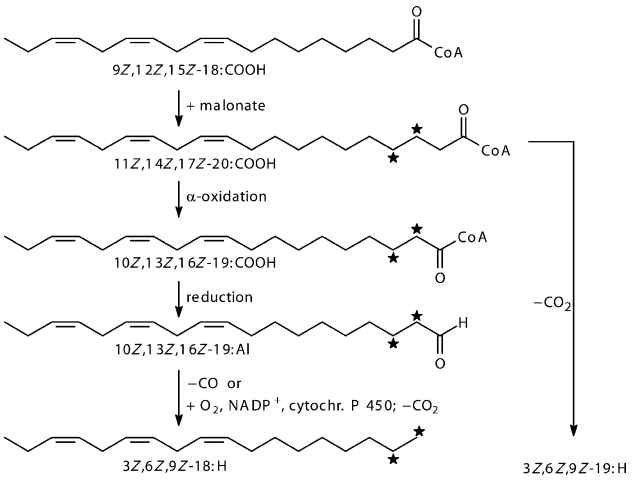


Fig. 1 Proposed biosynthetic pathway for the formation of 3*Z*,6*Z*,9*Z*-18:H based on the results of treatment of female moths with [2,2,3,3-$^2H_4$]-10*Z*,13*Z*,16*Z*-19:COOH and [3,3,4,4-$^2H_4$]-11*Z*,14*Z*,17*Z*-20:COOH. *Stars* indicate the positions of the deuterium labels

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the pheromone models of other bark beetles could benefit from systematic reexamination using newer technologies. Additionally, baits fortified with (+)-*endo*-brevicomin may enhance pest management strategies that exploit attractants for *D. frontalis*.

Keywords Attractant · Synergist · Bark beetle · GC-EAD · Scolytinae · Semiochemical · Sexual dimorphism · Aggregation pheromone · Coleoptera

Introduction

The pheromone system of the southern pine beetle, *Dendroctonus frontalis*, Zimmermann (Coleoptera: Curculionidae: Scolytinae), an aggressive tree-killer of *Pinus* spp. in southern North America and Central America, has been studied since the mid-1960s (reviewed in Smith et al. 1993). According to the model proposed by Renwick and Vité (1969) and subsequently refined (Vité and Francke 1976; Payne 1980; Smith et al. 1993), female beetles (the "pioneer" sex) initiate colonization of new host trees by boring into the bark while releasing the aggregation pheromone components frontalin and *trans*-verbenol. Frontalin is considered the major component of the aggregation pheromone, based both upon its ability to attract conspecifics in the absence of other compounds and the failure of any compound or combination to attract *D. frontalis* in its absence (Smith et al. 1993; Skillen et al. 1997). A blend of frontalin, *trans*-verbenol, and *alpha*-pinene (released by the host) attracts both sexes: males that pair with established females and females that initiate their own entrances in the bark. Attraction of sufficient numbers of conspecifics results in a lethal "mass attack" in which the host's constitutive resin defenses become so compromised that beetles can feed and reproduce unimpeded in the phloem. Arriving males produce verbenone and *endo*-brevicomin, compounds thought to interrupt attraction to the aggregation pheromone once the host is fully colonized and cause termination of mass attack.

Evidence that male-produced *endo*-brevicomin and verbenone function as antiaggregation pheromones includes: (1) production solely or predominantly by the second-arriving sex (Pitman et al. 1969; Hughes 1973), (2) the ability to inhibit attraction of *D. frontalis* to traps baited with frontalin plus *trans*-verbenol and/or host-compounds (Renwick and Vité 1970; Vité and Renwick 1971; Payne et al. 1978; Salom et al. 1992), and (3) the ability of devices releasing these compounds to reduce the numbers of beetles landing on hosts (Payne et al. 1977; Payne and Richerson 1979) or stop the expansion of individual *D. frontalis* infestations (Payne et al. 1992). However, Billings (1985) reported that low release rates of *endo*-brevicomin and verbenone enhanced attraction of flying *D. frontalis*. Subsequently, Vité et al. (1985) demonstrated that pure (+)-*endo*-brevicomin increased catches of *D. frontalis* in traps baited with frontalin and turpentine, whereas the (−)-enantiomer had an antagonistic effect.

The true function of *endo*-brevicomin in the biology of *D. frontalis* has remained unclear for at least two reasons. First, conflicting evidence exists regarding the enantiomeric composition of *endo*-brevicomin (Redlich et al. 1987; Grosman et al. 1997), hence the relative amounts of the apparently repellant (−) and the attractive (+)-enantiomers produced by *D. frontalis* is uncertain. Second, addition of male *D. frontalis* to female-infested pine bolts failed to alter responses of conspecifics (Coster et al. 1977; Svihra 1982), suggesting that male-produced semiochemicals neither inhibit nor enhance aggregation during host colonization.

In this paper, we reexamine the role of male beetles in semiochemical-mediated host colonization by *D. frontalis*. We assayed whether male beetles could alter attraction of conspecifics to female attacks when living pines were used as the host substrate rather than

pine bolts as in earlier studies. We then performed electroantennogram studies with volatiles derived from both newly emerged and mining *D. frontalis* to identify potential male-produced aggregation pheromone components, and we compared olfactory sensitivity for (+)-*endo*-brevicomin, a candidate male-produced aggregation pheromone component, against its antipode and the enantiomers of frontalin. We examined the influence of both feeding and pairing on the quantity and enantiomeric composition of *endo*-brevicomin produced by male *D. frontalis* and performed field trapping bioassays to determine the behavioral activity of (+)-*endo*-brevicomin and elucidate its likely function in host colonization.

Materials and Methods

Experimental Insects Adult *D. frontalis* were collected in a rearing box fitted with a refrigerated insect trap (Browne 1972) as they emerged from bark or bolts taken from naturally infested trees cut in the National Forests of Mississippi and Alabama in 2002–2006. Beetles were held for up to 1 week at 4°C in plastic Petri plates lined with pieces of moistened Kimwipe® (Kimberly-Clark, Roswell, GA, USA). Beetles were sexed by the mycangial bulge on the female pronotum and the deep median groove on the male frons (Wood 1982). Only undamaged beetles were used in experiments.

Attraction of D. frontalis to Artificially Infested Trees Within a mixed pine/hardwood stand that had a suboutbreak population of *D. frontalis* (Homochitto National Forest, Mississippi, 31.5° N, 91.2° W), we selected pairs of adjacent, apparently healthy loblolly pines, *Pinus taeda* L. Trees in each pair were 15–40 cm DBH (diameter breast height), located >100 m from any other pair, and >100 m from the nearest *D. frontalis*-infested tree. A saran screen cage (32 mesh, 1.0 m tall) was wrapped around the trunk of each tree at a height of 1–1.5 m. Access to the cage interior was through a Velcro® closure that ran the length of the cage; the top and bottom of the cage were sealed tightly to the bark with a 10-cm-wide strip of duct tape. To preclude beetle entry or escape through bark fissures, the outer bark was smoothed with a draw-knife where the top and bottom of the cage were taped to the tree. To prevent attacks by wild beetles, all noncaged portions from the soil to base of the crown were wrapped with clear polyethylene sheeting (6-ml thickness) secured with duct tape. Pines closer than 10 m to the caged trees were either removed or had their boles wrapped in sheeting to prevent "spillover" attacks. To promote successful attack of the trees by caged beetles, the primary resin defenses of each tree were challenged by stripping a 10-cm-wide girdle of bark immediately below the cages during the day before and again on the day of introduction of female beetles to the cages.

On one tree of each pair, 100 *D. frontalis* females were sealed into the cage, and then, three nonbaited, 12-unit multiple funnel traps (Chemtica International, San Jose, Costa Rica) were suspended against the outside of the cage equidistant around the tree circumference. On the following day, trap catch was collected, and then, either 100 males or no further beetles were added to the cage, as determined by a coin toss. Beetles were collected again 2 and 4 days later. Peak attraction to naturally infested trees and artificially infested logs occurs 2–3 days after initiation of attacks (Coster and Vité 1965; Coster et al. 1977). After the final trap collection, infested trees were immediately felled, and the caged portions were removed for dissection in the laboratory. Approximately 1 week after felling of the first tree of each pair, the entire procedure was repeated on the second tree except that it received the opposite treatment with regard to the introduction of males. In total, eight

pairs of trees were assayed between 20 June and 1 August 2006. We used paired *t* tests to identify treatment effects on trap catch and numbers of attacks on trees. The proportions of males trapped per tree were normalized with $arcsin\sqrt{x}$, and treatments were compared with Student's *t* test. Bonferroni's adjustment (α=0.017) was applied to comparisons of catch within the three trapping intervals.

Electrophysiological Analyses We examined relative olfactory sensitivities of *D. frontalis* to volatile compounds isolated from males. We quantitatively analyzed coupled gas chromatography-electroantennographic detection (GC-EAD) responses of beetles to 11 male-produced compounds that were shown in a previous, qualitative study to have olfactory activity (Sullivan 2005). In that study, single antennae from male and female *D. frontalis* were exposed to 0.1 beetle equivalents of the mid/hindgut extract of 100 newly emerged male *D. frontalis.* We also performed new GC-EAD analyses on the antennae of ten male and ten female *D. frontalis* exposed to volatiles from male beetles that had been paired with a female in a pine bolt <1 day (extraction treatment 3, below). The pooled pentane extract from 15 males was concentrated until approximately 1 μl equaled 0.2 beetle equivalents, and this quantity was injected into the GC, thus delivering 0.1 beetle equivalents to the antennal preparation (the effluent was split 1:1 between the GC's flame ionization detector [FID] and the antennal preparation). Otherwise, the GC-EAD apparatus and procedures were identical to those published by Sullivan (2005). For both GC-EAD data sets, raw response voltages for all compounds that produced an EAD deflection with at least four antennae were log transformed and then analyzed by repeated-measures analysis of variance (ANOVA) and Tukey's pairwise tests (α=0.05; SPSS 1997).

Because *endo*-brevicomin consistently produced greater antennogram responses than any other male-derived volatile, we generated dose–response curves to compare the relative olfactory sensitivity of *D. frontalis* to the separated enantiomers of both *endo*-brevicomin and the female-produced aggregation pheromone component frontalin. Antennae were exposed to mixtures containing tenfold dilutions of frontalin and *endo*-brevicomin (approximately 1000, 100, 10, 1, 0.1, 0.01, and 0.001 ng/μl of each enantiomer delivered to the antennae) as well as the EAD-active internal standard (−)-verbenone at a fixed concentration (100 ng). Each experimental replicate consisted of a single GC-EAD run with a fresh antennal preparation and one of the dilutions assayed in random order. Enantiomers were separated on a GammaDex-225 column (30 m×0.25 mm×0.25 μm film; Supelco, Bellefonte, PA, USA) by using the temperature program 5°/min from 60 to 117°, then 45°/min to 225° held 5 min. This program produced a 0.18- to 0.28-min separation between the GC peaks of both the frontalin and *endo*-brevicomin enantiomers and a >3.0-min separation between peaks of the three compounds (Fig. 1). We were unable to identify a chiral column phase that reversed the elution order of the enantiomers of frontalin and *endo*-brevicomin, hence all GC-EAD runs were performed with (+)-frontalin and (−)-*endo*-brevicomin eluting ahead of their respective antipodes. To ensure that recent exposure to the antipode was not reducing antennal responses to the second-eluting enantiomer, GC-EAD runs were performed both with synthetically purified, single enantiomers (Phero Tech Inc., Delta, BC, Canada; 94–97% enantiomeric purity; two to four replicates per dilution) as well as racemic mixtures (four replicates per dilution) of frontalin and *endo*-brevicomin. Mean response voltages did not differ significantly between these data sets at any concentration (nonparametric *t* test, α=0.05, Microsoft® EXCEL 2002); therefore, the data were pooled. To minimize variation as a result of inconsistent quality of the antennal preparations, response voltages to frontalin and *endo*-brevicomin enantiomers were computed relative to response voltage to the internal standard, (−)-verbenone. The threshold of detection for each

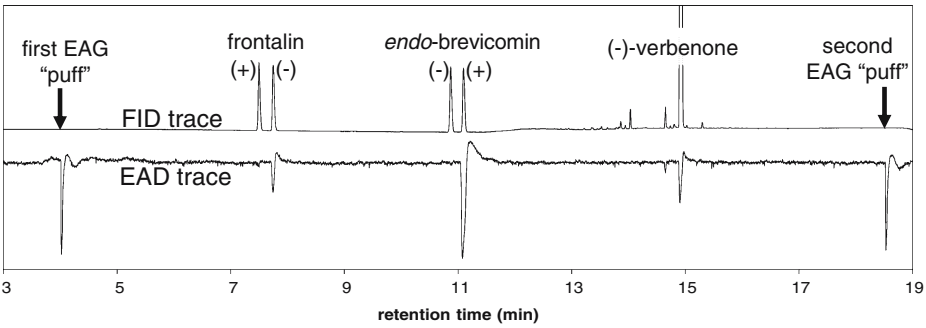


Fig. 1 GC-EAD trace from a male *D. frontalis* antenna exposed to one of the doses (10 ng) used to calculate dose–response curves for *D. frontalis* sensitivity to the enantiomers of frontalin and *endo*-brevicomin. The *upper* trace is from the GC's flame ionization detector (*FID*), while the *lower* trace is from the electroantennographic detector (*EAD*). Enantiomers were separated on a *gamma*-cyclodextrin-phase capillary GC column. Verbenone was included in each injected dilution as an electrophysiologically active internal standard, and an electroantennogram (*EAG*) stimulus (consisting of a blend of *D. frontalis* semiochemicals in a pasteur pipette) was "puffed" over the antenna at the beginning and at the end of each GC-EAD run to estimate decline in overall responsiveness of the antennal preparation during this interval

enantiomer was calculated as the lowest concentration for which the EAD deflection voltage exceeded the 90th percentile of the height of background noise on at least three out of eight runs ($P<0.038$, table of cumulative binomial probabilities; Bhattacharyya and Johnson 1977). Mean and standard error of responses to enantiomers were calculated for concentrations that exceeded the threshold of detection. To calculate the actual concentration of each enantiomer exposed to the antenna, the integration area of each FID peak during 0.2 s at maximum peak height was divided by the total integration area, multiplied by the known total quantity of enantiomer eluting, and then divided by the volume of air transferring the effluent to the antenna every 0.2 s (i.e., 1.33 ml).

For all GC-EAD data sets, the heights of signal voltage deflections in each GC-EAD run were corrected for the inevitable loss of responsiveness in the antennae that occurred over the duration of the run. This correction was accomplished by measuring the decline in electroantennogram (EAG) response to a uniform stimulus (a mixture of *D. frontalis* semiochemicals delivered from a Pasteur pipette) "puffed" over the antenna at the beginning and end of each GC-EAD run (Fig. 1). The percent reduction in height of the response to the second EAG stimulus relative to the first was assumed to be a linear function of time and to affect all stimulants equally.

Pheromone Isolation and Analysis We used procedures described by Sullivan (2005) and determined the influence of feeding and pairing on the quantities of *endo*-brevicomin, frontalin, verbenone, and myrtenol produced by one or both sexes of *D. frontalis*. We sampled volatiles from individual beetles that either (1) were newly emerged (i.e., had appeared in the rearing box during the previous 3 days), (2) had been mining singly in a freshly cut loblolly pine bolt for <1 day, or (3) had been mining in a bolt with a mate for <1 day (females had initiated galleries 1 day before the introduction of males). To obtain attacks on bolts, we confined beetles within gelatin capsule halves secured over 1-mm-diameter holes drilled into the bark. Beetles that produced frass in treatments 2 and 3 were carefully dissected from the bark and kept up to 4 h at 0–4°C before headspace sampling. To collect volatiles from beetles, they were held singly for 16–20 h at room temperature in static headspace sampling enclosures (Sullivan 2005) containing ~3 mg of Super Q

adsorbent (Alltech, Deerfield, IL, USA). Afterward, the Super Q and the excised mid/hindgut of each beetle were extracted together in a total of 100 μl pentane spiked with 180 ng of the internal standard heptyl acetate. In one experiment, 31–34 individuals per treatment per sex were sampled from a single pool of beetles derived from infestations in the Chickasawhay National Forest of southeastern Mississippi (31.5° N, 89.0° W). In addition, we checked for possible regional variation in the enantiomeric ratios of *endo*-brevicomin by sampling additional males in treatment 3 above from both the Homochitto National Forest in western Mississippi (31.4° N, 91.1° W; N=4) and the Talladega National Forest of Alabama (32.9° N, 87.1° W, N=11).

The samples were analyzed on a Hewlett-Packard 6890-5973 coupled GC/mass spectral detector (GC-MSD) with helium as the carrier gas. Two microliters of sample were injected in splitless mode and analyzed with both nonchiral phase (INNOWax; 60 m×0.25 mm×0.25 μm film; Agilent Technologies, Wilmington, DE, USA) and chiral phase (Gamma-Dex-225; 30 m×0.25 mm×0.25 μm film; Supelco) capillary GC columns. For nonchiral GC-MS runs, the oven program was 40°C for 1 min, 16°C/min to 80°C, then 7°C/min to 230°C; for chiral runs, it was 40°C for 1 min, 5°C/min to 70°C, 2°C/min to 155°C, then 25°C/min to 220°C, or alternatively, 40°C for 1 min, then 5°C/min to 220°C. Analytes were quantified in the nonchiral runs by using response curves calculated from analyses of a dilution sequence of known quantities of compounds. Quantitative differences among treatment groups within sex were analyzed with a Kruskal–Wallis ANOVA followed by Dunn's test for pairwise comparisons (α=0.05; SPSS 1997).

Enantiomers of frontalin and *endo*-brevicomin in the samples were identified by retention time matches on the chiral column with synthetic standards of these enantiomers supplied by Phero Tech. We confirmed the optical rotation of the chiral *endo*-brevicomin standards by polarimetry. Enantiomeric ratios of frontalin and *endo*-brevicomin were calculated by examining single ion chromatograms of diagnostic ions for each compound (100 and 114 amu, respectively) and then computing the ratio of the integration areas of peaks at the confirmed retention times of the (+)- and (−)-enantiomers. Response of the MSD had been found to be essentially linear through the range of enantiomer concentrations examined.

Attraction of D. frontalis to Synthetic Baits We evaluated the behavioral responses of *D. frontalis* to synthetic (+)-*endo*-brevicomin baits by using 12-unit multiple funnel traps erected within the same stand used for the artificially infested tree experiment during a period when it was experiencing a declining outbreak of *D. frontalis*. Traps were located >100 m apart, >10 m from the nearest susceptible pine, and >1 km from the nearest known multiple-tree *D. frontalis* infestation. They were suspended from metal poles with the bottom funnel ~1 m above the ground. Bait bioassay 1 (12 replicates; 18 September–17 October 2005) compared four bait treatments: pine turpentine; turpentine and (+)-*endo*-brevicomin; turpentine and racemic frontalin; (+)-*endo*-brevicomin, racemic frontalin, and turpentine. Bait bioassay 2 (nine replicates; 17 January–1 March 2006) compared five bait treatments: unbaited trap; racemic frontalin; (+)-*endo*-brevicomin; racemic frontalin and (+)-*endo*-brevicomin; (+)-*endo*-brevicomin, racemic frontalin, and turpentine.

The frontalin bait consisted of two, 400-μl-capacity LDPE microcentrifuge tubes containing racemic frontalin (>95% purity, Chemtica International). Turpentine (steam distilled from loblolly pine; Hercules Inc., Brunswick, GA, USA) was released from a 250-ml-capacity brown glass bottle with a piece of 1 cm diameter cotton dental wick immersed in the turpentine and protruding 2.5 cm through the cap. The (+)-*endo*-brevicomin was synthesized by the combination of procedures reported by Mori and Seu (1985) and Mori

and Kiyota (1992) that employs lipase-catalyzed desymmetrization of a diol and Wacker oxidation of an olefin and was >99% enantiomerically and >95% chemically pure. It was released from a 1.17-mm i.d. glass capillary sealed at one end and suspended open-end up inside an inverted 2-ml glass vial. The frontalin and *endo*-brevicomin baits were suspended at the fourth funnel from the bottom of the trap, whereas the turpentine bottle was placed within the funnel immediately below the trap top (to protect the exposed wick from rain). Release rates were measured for all baits within a fume hood at 23±2°C, either gravimetrically (turpentine, 7 g/day; frontalin, 5 mg/day) or by loss of volume in the capillary (*endo*-brevicomin, 0.2 μl/day=0.2 mg/day). Trap cups were filled with a few centimeters of propylene glycol and water, and catch was collected every 1–2 weeks. Traps were arranged into three lines with one trap per treatment and 150±50 m between traps. Treatments were assigned at random within lines and rerandomized without replacement to any previous position each time catch was collected. Each rerandomization/collection per trap line was one complete statistical block (=replicate). Raw catches of male and female *D. frontalis* were transformed with $log(X+0.5)$ and analyzed for significant differences among treatment means by using a two-way ANOVA (factors were treatment and block) and Tukey's pairwise comparisons ($\alpha=0.05$; SPSS 1997). For bait treatments that trapped more than one insect, the proportion of males trapped per replicate was transformed ($arcsin\sqrt{x}$), and treatments were compared with Student's *t* test.

Results

Attraction of D. frontalis to Artificially Infested Trees In the first 2 days after introduction of males, trees caged with both sexes of *D. frontalis* attracted significantly greater numbers of conspecifics than trees caged with females alone (Fig. 2). This difference disappeared during the next 2 days. Beetle catch summed for all 4 days after introduction of males was likewise significantly higher for trees infested with both sexes (paired *t* test; $P<0.001$). For this same period, sex ratio (M/F) of beetles trapped at trees with both sexes (1.2:1), and females only (1.6:1) did not differ significantly (*t* test; $P=0.82$). Dissections of the caged portions of trees revealed that both treatments had similar numbers of attacks initiated into the phloem (female only, 56.0±2.4; both sexes, 59.6±4.5; paired *t* test, $P=0.37$) and

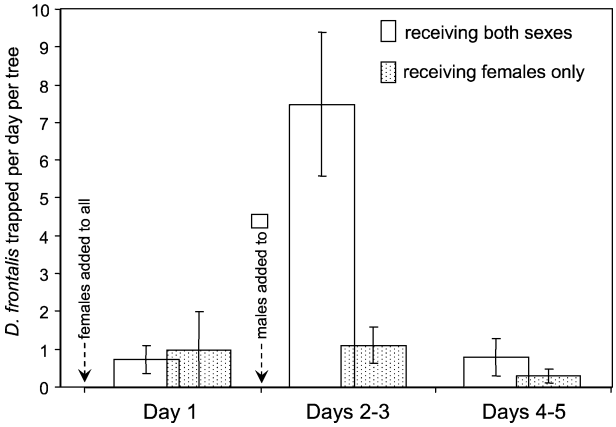


Fig. 2 Mean catches (±SE) of *D. frontalis* in traps affixed to live pines artificially infested either with conspecific females or with both sexes. Catch was significantly different between treatments on days 2–3 but not day 1 or days 4–5 (Paired *t* test, $P<0.017$)

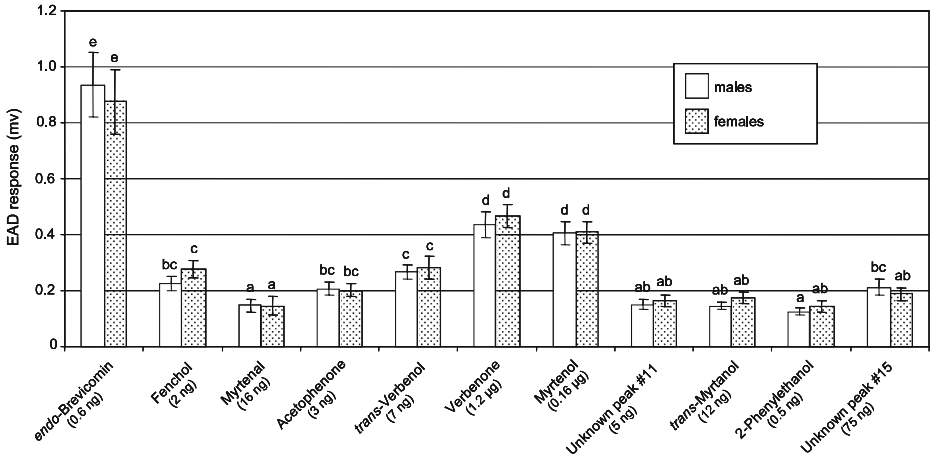


Fig. 3 Mean (±SE) EAD response by antennae of *D. frontalis* exposed to 0.1 insect equivalents of pooled gut extract of 100 newly emerged male *D. frontalis* (Sullivan 2005). Within sex, means associated with the same *letter* were not significantly different (α=0.05, Tukey test). The approximate quantity of each compound exposed to the antenna is given in *parentheses*

confirmed that, in trees that had received both sexes, a substantial number of males had successfully paired with females in galleries (30.6±4.6). Greater numbers of galleries with live, solitary females were found in female-only trees (18.4±2.6) compared to trees receiving both sexes (5.3±1.5; paired *t* test P=0.005). Live, solitary females had mined in the phloem an average of 3.73±0.16 cm, whereas live pairs had mined 7.81±0.22 cm (nonparametric *t* test, P<0.001).

Electrophysiological Analyses In GC-EAD analyses of 0.1 insect equivalents of extract from newly emerged *D. frontalis* males, antennae of both sexes responded with

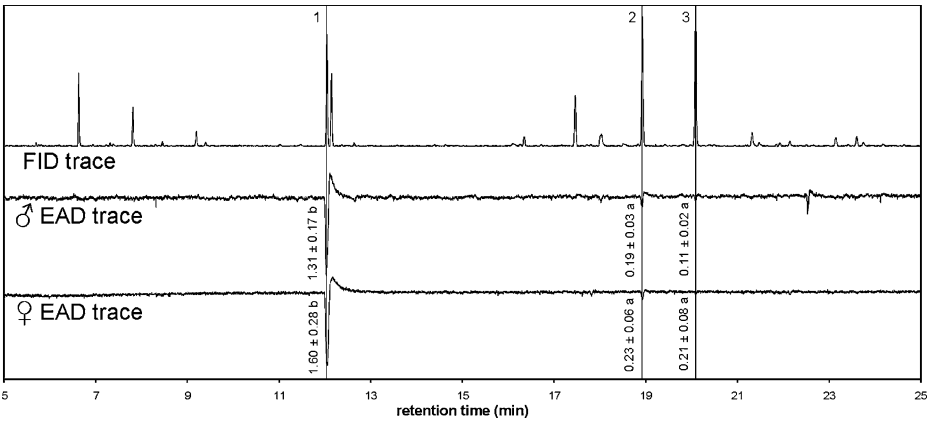


Fig. 4 GC-EAD traces of individual *D. frontalis* exposed to 0.1 insect equivalents of the pooled aerations/extracts of 15 male *D. frontalis* that had been paired in a pine bolt with a female beetle for approximately 1 day. *Peaks* detected by the flame ionization detector (*FID*) that consistently coincided with an EAD deflection from either sex are numbered: *endo*-brevicomin, *1*; verbenone, *2*; myrtenol, *3*. Values adjacent to each EAD peak represent the mean (±SE mv) response of ten sampled antennae. Within sex, means associated with the same *letter* were not significantly different (Tukey test; α=0.05)

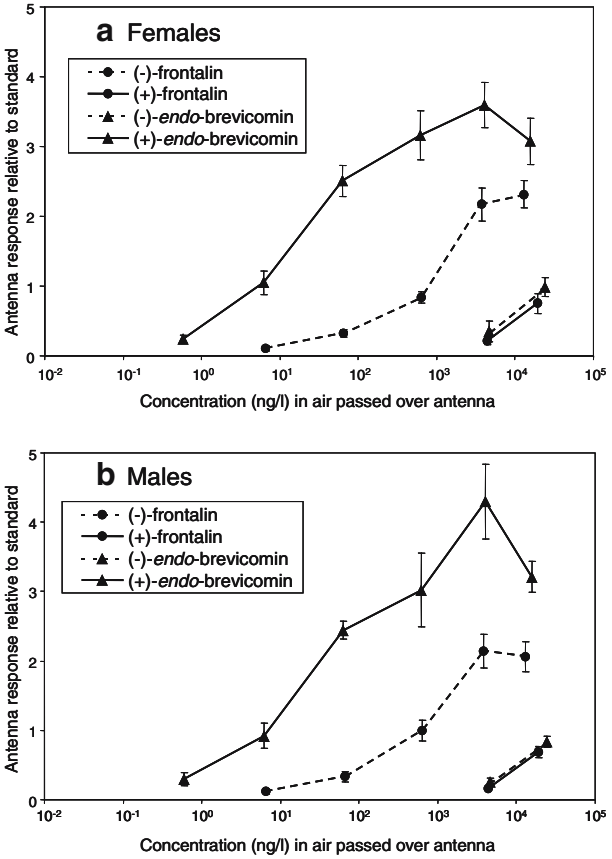


Fig. 5 Dose–response curves of EAD responses of female (**a**) and male (**b**) *D. frontalis* to the enantiomers of frontalin and *endo*-brevicomin resolved on a *gamma*-cyclodextrin-phase capillary column. *Data points* (mean response±SE) are shown only for concentrations for which the antennae registered a response that was significantly above the level of background noise (see text); hence, the furthest *leftward* point of each plot represents the response threshold for the enantiomer. The lowest concentration tested was 0.06 ng/l

significantly higher voltages to *endo*-brevicomin than any other olfactory stimulant (Fig. 3). We found no olfactory stimulants in volatiles sampled from feeding, paired males that were not also produced by newly emerged individuals. When 0.1 insect equivalents of pooled volatiles from paired, mining males were analyzed by GC-EAD, only three compounds produced consistent responses from the antennae: *endo*-brevicomin, verbenone, and myrtenol (Fig. 4). *endo*-Brevicomin elicited significantly greater voltages from the antennae than either of the other two olfactory stimulants from paired males.

Exposure of *D. frontalis* antennae by chiral GC-EAD to a logarithmic progression of doses of the enantiomers of frontalin and *endo*-brevicomin revealed that, for both sexes, the threshold of detection for (+)-*endo*-brevicomin was four orders of magnitude lower than for either (−)-*endo*-brevicomin or (+)-frontalin and one order of magnitude lower than for (−)-frontalin (Fig. 5). The threshold of detection for (−)-frontalin was three orders of magnitude lower than for its antipode. Through the entire range of concentrations tested, the mean response voltage elicited in the antennae was apparently higher for (+)-*endo*-brevicomin than for either enantiomer of frontalin or for (−)-*endo*-brevicomin.

Pheromone Isolation and Analysis Significant quantities of frontalin were detected from females and *endo*-brevicomin from males, whether newly emerged, mining singly, or mining in pairs (Fig. 6a). With a threshold of detection of approximately 1 ng per beetle, frontalin was detected in only six of 110 male beetles sampled. These six came from all

three treatment categories, and with one exception (9.5 ng from a male mining singly), the quantity detected was <3 ng. Similarly, traces of *endo*-brevicomin (1–3 ng) were detected in only seven of 96 females sampled. In contrast, *endo*-brevicomin was detected in 90% of emergent males, 81% of solitary mining males, and 100% of paired males; frontalin was detected in 100% of sampled females.

The quantity of frontalin detected from females excised while mining in a pine bolt was significantly (ninefold) higher than from newly emerged females (Fig. 6a). Less frontalin was detected from paired, mining females than females mining singly; however, these paired females had been in the bolt 1 day longer than the solitary ones, hence the relative effects of pairing vs time spent mining could not be distinguished. Males that had paired with a female in a gallery produced higher quantities of *endo*-brevicomin than males that were either newly emerged (15-fold) or mining singly (fivefold), whereas males mining singly did not differ significantly from newly emerged beetles (Fig. 6a). Peak recorded production of frontalin by females (i.e., females mining solitary, 547±63 ng per beetle) was nearly twice the peak production of *endo*-brevicomin by males (i.e., males mining paired, 291±55 ng per beetle). In contrast to *endo*-brevicomin, production of both verbenone and myrtenol were higher in newly emerged males than in those that were mining in a bolt either alone or paired, and the presence of a female did not significantly alter the production of these monoterpenes by mining males (Fig. 6b).

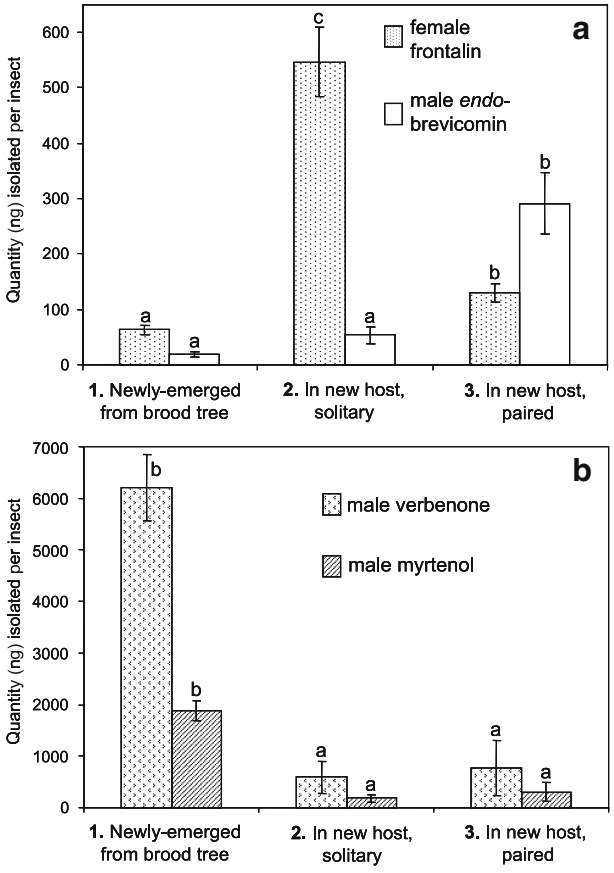


Fig. 6 Mean quantities (±SE) of volatiles isolated from individual *D. frontalis*: the bicyclic ketals frontalin and *endo*-brevicomin from females and males, respectively (**a**) and the monoterpenes verbenone and myrtenol from males (**b**). Beetles (N=31–34) either (*1*) had emerged within the previous 24 h from portions of a naturally infested pine, (*2*) had been infested onto a fresh pine bolt 1 day earlier, or (*3*) had been paired with a mate in a pine bolt for 1 day. In treatment 3, females had been allowed to initiate a nuptial chamber for 1 day before introduction of male beetles and thus had been in the bolt 2 days when sampled. Within sex/compound, *bars* associated with the same *letter* were not significantly different (Dunn's test, α=0.05)

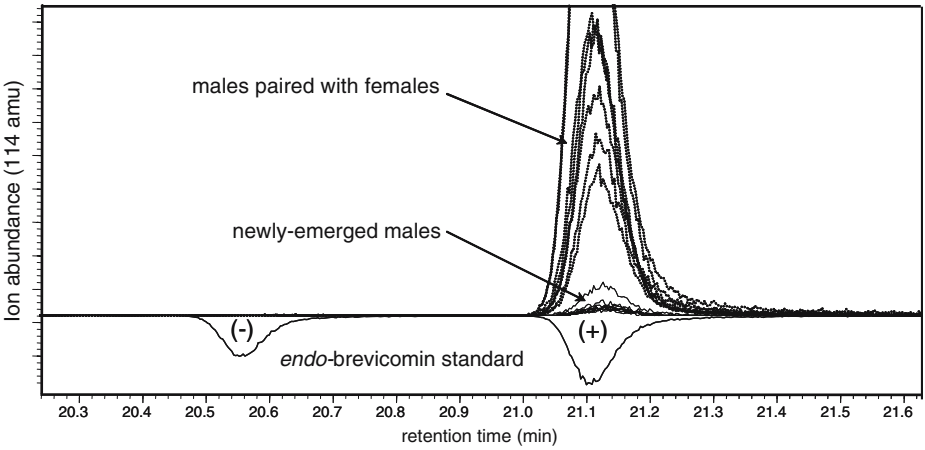


Fig. 7 Superimposed GC-MS single-ion chromatograms of volatiles from male *D. frontalis* resolved on an enantioselective GC column. Shown are eight traces each of aerations/extracts of either newly emerged males (positive traces, *solid lines*) or males paired with a female in a pine bolt <1 day (*dotted lines*), as well as a retention time standard containing a 1:2 ratio of (−):(+) *endo*-brevicomin (*inverted trace*)

Only the (+)-enantiomer of *endo*-brevicomin was isolated from male beetles, irrespective of beetle treatment category or source population (Fig. 7). Because the amount of (−)-*endo*-brevicomin never exceeded the MSD's threshold of detection in any sample, the mean enantiomeric ratio was not less than 86.5% (+):13.5% (−)±2.2% [i.e., the mean ratio of the quantity of (+)-*endo*-brevicomin to the threshold of detection observed in newly emerged males] for any treatment group or sampled population (Table 1). This lower limit of confidence is probably conservative given the fact that the ratio of (+)-*endo*-brevicomin to the threshold of detection exceeded 99:1 [i.e., >99% (+)-*endo*-brevicomin] in 18 samples. For female samples where both enantiomers of frontalin exceeded the threshold of detection (88 of 96), the mean enantiomeric ratio was 95.4% (−): 4.6% (+)±0.7% SD. Feeding and/or pairing had no significant effect on the enantiomeric ratio of female frontalin (Kruskal–Wallis ANOVA, H=0.4843, df=2, P=0.79).

Attraction of D. frontalis to Synthetic Baits In bait bioassay 1 (Fig. 8), traps baited with turpentine, (+)-*endo*-brevicomin, and frontalin caught significantly more *D. frontalis* of either sex than turpentine either alone or paired with either frontalin or *endo*-brevicomin. Neither turpentine alone nor the combination of turpentine and (+)-*endo*-brevicomin attracted any *D. frontalis*, whereas turpentine combined with frontalin attracted greater numbers of males (and total beetles; P=0.014) than these treatments. Addition of (+)-*endo*-brevicomin to frontalin/turpentine-baited traps resulted in a drop in the sex ratio of the responding beetles (M/F) from 3.4:1 to 1.3:1 (P=0.032). In bait bioassay 2 (Fig. 9), traps baited with the combination of turpentine, (+)-*endo*-brevicomin, and frontalin caught significantly more *D. frontalis* of either sex than traps either lacking baits or baited with frontalin and/or (+)-*endo*-brevicomin in the absence of turpentine. Traps baited with (+)-*endo*-brevicomin alone failed to attract more beetles than nonbaited traps, whereas traps baited with frontalin alone attracted more females (and total beetles; P=0.024) than nonbaited traps. Traps baited with frontalin and (+)-*endo*-brevicomin did not differ significantly in catches from either nonbaited traps or traps baited with frontalin alone. The sex ratios (M/F) trapped by frontalin alone (1.6:1), frontalin/(+)-*endo*-brevicomin (1.1:1),

Table 1 Enantiomeric ratio of *endo*-brevicomin in volatiles collected from male *D. frontalis* as the minimum possible percentage of the (+)−enantiomer

Beetle Origin	Beetle Condition		
	Newly Emerged	Mining Singly	Mining Paired
31.5° N, 89.0° W	>86.5[a]±2.2%[b] (27)[c]	>92.3±1.9% (22)	>98.6±0.4% (31)
32.9° N, 87.1° W	n/a	n/a	>98.7±0.2% (11)
31.4° N, 91.1° W	n/a	n/a	>98.6±0.2% (4)

[a] Mean ratio (as a percentage) of the abundance of the (+)-enantiomer to the threshold of detection of the MSD. Since (−)-*endo*-brevicomin was not detected, its greatest possible quantity in any sample was less than the threshold of detection.

[b] Standard error of the mean

[c] Sample size (*N*)

and frontalin/(+)-*endo*-brevicomin/turpentine (1.8:1) did not significantly differ ($P>0.18$); however, low numbers trapped by the turpentine-lacking treatments gave insufficient power to these comparisons.

Discussion

Our data provide the first direct evidence that *D. frontalis* males can enhance the aggregation of conspecifics to pines undergoing colonization (Fig. 2), implying that mass attack is mediated by pheromone components from both sexes. Thus *D. frontalis* resembles congeners *D. adjunctus* Blandford (Hughes et al. 1976), *D. brevicomis* LeConte (Wood and Bedard 1977), and *D. ponderosae* Hopkins (Borden et al. 1987) that have been shown to aggregate on pines in response to pheromone components from both sexes. However, two previous studies showed that pine bolts artificially infested with *D. frontalis* pairs were no more attractive to conspecifics than those infested solely with females (Coster et al. 1977; Svihra 1982). Whereas our girdling of experimental trees likely improved the chances of successful beetle attack and establishment (Tisdale et al. 2003), the defensive responses of these trees remained strong, as evidenced by pitch tubes at most beetle entrance holes and

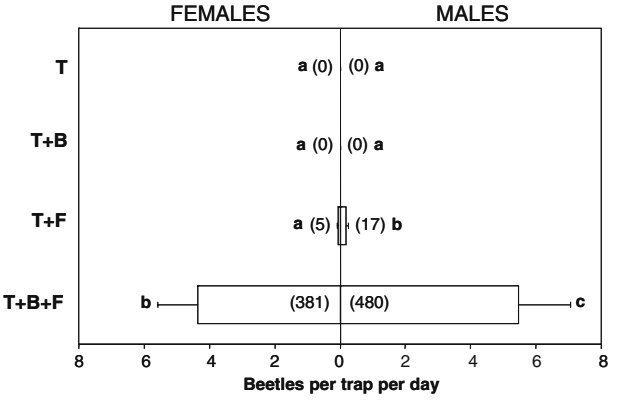


Fig. 8 Bait bioassay 1: Mean (+SE) catch of *D. frontalis* in multiple-funnel traps baited with loblolly pine turpentine (*T*), (+)-*endo*-brevicomin (*B*), and/or racemic frontalin (*F*). Within sex, *bars* associated with the same *letter* were not significantly different (Tukey test, $\alpha=0.05$). Total catch for each treatment/sex is given in *parentheses*

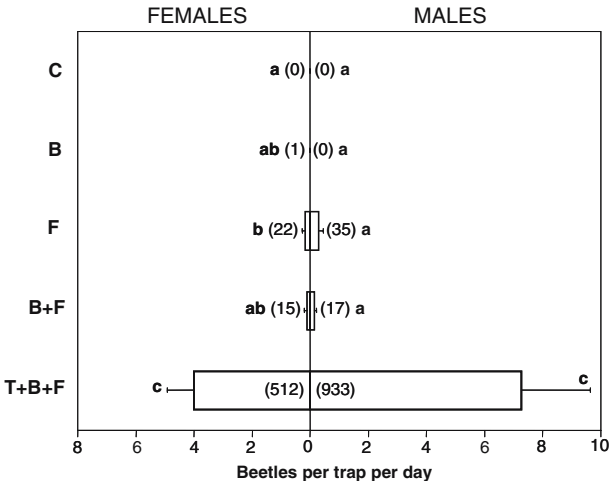


Fig. 9 Bait bioassay 2: Mean (+SE) catch of *D. frontalis* in multiple-funnel traps baited with loblolly pine turpentine (*T*), (+)-*endo*-brevicomin (*B*), and/or racemic frontalin (*F*) or nothing (*C*). Within sex, *bars* associated with the same *letter* were not significantly different (Tukey test, α=0.05). Total catch for each treatment/sex is given in *parentheses*

numerous beetles found "pitched" into their galleries. In contrast, pitch tubes rarely occur at entrances when beetles attack pine bolts (personal observation). Bolts cut from trees recently infested with *D. frontalis* rapidly lose attractiveness to conspecifics, and high host moisture content and resin exudation have been associated with enhanced aggregation by *D. frontalis* and other *Dendroctonus* spp. (Vité and Renwick 1968; Vité and Pitman, 1968). High resin production may delay beetle pairs from extending their galleries into the phloem and thus prolong pheromone release near the entrances. *D. frontalis* will sometimes attack and reproduce in dead or dying hosts, such as felled trees, that are presumably similar in quality to bolts (Moser et al. 1987); however, during outbreaks, they predominantly attack healthy trees. Hence, our infested-tree study may provide a more accurate picture of pheromone dynamics for outbreak-phase *D. frontalis*.

Bark beetles typically possess disproportionate olfactory sensitivity to components of their aggregation pheromones, which function over large distances and at low concentrations (Dickens 1979; Whitehead 1986; Smith et al. 1988). The exceptionally strong electrophysiological responses that (+)-*endo*-brevicomin elicited in the antennae of both sexes of *D. frontalis* singled-out this compound as a likely aggregation pheromone component (Figs. 3, 4, and 5). This result was also consistent with evidence that only the (+)-enantiomer of *endo*-brevicomin is attractive to *D. frontalis* (Vité et al. 1985). Dickens and Payne (1977) reported that among seven known semiochemicals for *D. frontalis*, *endo*-brevicomin ranked second only to frontalin in the percentage of olfactory acceptor sites occupied during odorant saturation.

Vité and Renwick (1971) reported that *endo*-brevicomin was produced merely in "subnanogram quantities by certain males" and therefore questioned whether this compound was a true pheromone for *D. frontalis*. Grosman et al. (1997) isolated an average of 17 ng *endo*-brevicomin per male beetle from three geographically isolated *D. frontalis* populations. These earlier studies examined only newly emerged beetles (Pitman et al. 1969; Grosman et al. 1997), which according to our data, produce relatively small amounts of *endo*-brevicomin (Fig. 6a). In contrast, we isolated nearly 300 ng *endo*-brevicomin on average from paired males, an amount roughly comparable to the frontalin isolated from mining females.

Production of frontalin in females was stimulated by mining in the host, whereas *endo*-brevicomin production in males was stimulated by pairing (Fig. 6a). In contrast, other male-produced compounds such as verbenone and myrtenol declined in concentration once males

entered the bark (Fig. 6b; Sullivan 2005). The majority of such compounds (including verbenone, myrtenol, fenchol, myrtenal, acetophenone, *trans*-myrtanol, and 2-phenylethanol) are attractant antagonists (Payne et al. 1978; Sullivan 2005; Sullivan et al. 2007), and the separation in the timing of their production relative to *endo*-brevicomin is consistent with differing behavioral functions. This finding also suggests that synthesis and/or accumulation of *endo*-brevicomin is regulated by physiological mechanisms different from other male-produced compounds. In support of this inference, treatment of 5-day-old, callow adult male *D. frontalis* with either juvenile hormone II or the juvenile hormone analog methoprene stimulated an increase in hindgut concentrations of *endo*-brevicomin but not verbenone or myrtenol (Bridges 1982). Independent regulation of *endo*-brevicomin is also implied by evidence that bark beetle-produced bicyclic ketals like brevicomin and frontalin are synthesized de novo, whereas oxygenated monoterpenes such as verbenone and myrtenol may be derived from ingested or inhaled host monoterpenes (Hughes 1973; Seybold and Tittiger 2003).

Vité et al. (1985) first hypothesized that the complete aggregation pheromone of *D. frontalis* included male-produced (+)-*endo*-brevicomin, which they found to cause a fivefold increase in attraction to female-produced frontalin and host terpenes. Our data confirm their results (Fig. 8) while documenting a remarkable 40-fold enhancement effect from the addition of (+)-*endo*-brevicomin to a frontalin/host terpene bait. However, Vité et al. (1985) also showed that (−)-*endo*-brevicomin is an attractant antagonist, and there are conflicting data on the enantiomeric ratios of *endo*-brevicomin produced by *D. frontalis* males. Redlich et al. (1987) found approximately 97% of the (+)-enantiomer when they used enantioselective capillary GC-MS to analyze aerations and hindgut extracts of emergent male *D. frontalis*. Grosman et al. (1997) used enantioselective GC-FID to analyze gut extracts from emergent males from three different populations and reported a range of 9–21% (+) with no significant variation among regions. In contrast, our examination of aerations/extracts of male *D. frontalis* using enantioselective GC-MS failed to detect even trace quantities of (−)-*endo*-brevicomin, despite detecting the (+)-enantiomer at hundreds of times the detection threshold of the MSD (Fig. 7).

The disparity among these three data sets was most likely caused by differences in the reliability of the analytical techniques, because no significant geographic variation in the enantiomeric ratio of *endo*-brevicomin was identified in our data or those of Grosman et al. (1997). Our findings and those of Redlich et al. (1987) both indicated that the (+)-enantiomer was predominant, and both studies were performed by using MSD chromatograms of single, diagnostic ions for *endo*-brevicomin. This procedure would have largely ensured that only the target molecule was quantified and would have prevented overestimation of the abundance of (−)-*endo*-brevicomin because of coelution of compounds indistinguishable by FID alone, as could have occurred in the study of Grosman et al. (1997). Additionally, our study provided a more meaningful picture of *endo*-brevicomin chirality than previous ones, because it examined paired males, apparently the only life stage that produces this compound in large quantities. The greater olfactory sensitivity of both sexes for (+)-*endo*-brevicomin and (−)-frontalin than for the respective antipodes (Fig. 5; Payne et al. 1982) mirrors the greater production of these enantiomers by the insects. Thus, the totality of evidence indicates that *D. frontalis* is utilizing only (+)-*endo*-brevicomin, the attractive enantiomer as shown by Vité et al. (1985).

Among *Dendroctonus* spp., a pheromone component produced predominantly by one sex typically elicits the strongest attractive response from the opposite sex (Vité and Pitman 1969; Hughes et al. 1976; Borden et al. 1987; Payne et al. 1987). In concurrence, Vité et al.

(1985) observed that addition of male-produced (+)-*endo*-brevicomin to attractant-baited traps shifted the sex ratio (M/F) from 4.6:1 to 2.0:1. In our study, both the introduction of males to female-infested trees and the addition of (+)-*endo*-brevicomin to traps baited with frontalin either alone or with turpentine caused the male-biased sex ratio of attracted beetles to shift closer to a balance between the sexes. However, these sex ratio differences were statistically significant only in bait bioassay 1, possibly because of the low catch of beetles by at least one of the treatments in these various pairwise comparisons. Our GC-EAD studies showed that the sexes differed little in their olfactory sensitivity for (+)-*endo*-brevicomin (Fig. 5), indicating that any sexually dimorphic behavioral responses to (+)-*endo*-brevicomin are not reflected in sex-specific abundance of olfactory receptors.

The dramatic increase in beetle attraction observed when (+)-*endo*-brevicomin was added to frontalin/turpentine suggests that this compound is an important component of the *D. frontalis* aggregation pheromone and that it is critical in mediating successful mass attacks on host trees. However, in contrast to frontalin, (+)-*endo*-brevicomin appeared to be entirely unattractive to *D. frontalis* in the absence of other compounds (Fig. 9) and showed no ability to act synergistically with turpentine (Fig. 8; Billings 1985). Hence, the male and female aggregation pheromone components do not play equivalent or reciprocal roles. Curiously, (+)-*endo*-brevicomin exhibited no synergistic activity with frontalin alone (Fig. 9), but the low catch by these treatments gave inadequate statistical power to their comparison. *Dendroctonus* males are not known to initiate attacks; hence, the failure of *D. frontalis* to respond to male pheromone components alone or with host odors seems consistent with females' exclusive role as pioneers. Nonetheless, attraction to male-produced compounds in the absence of those from females has been documented in *D. adjunctus* (Hughes et al. 1976) and *D. terebrans* (Olivier; Payne et al. 1987; Phillips et al. 1990), and the male-produced compound frontalin can induce *D. ponderosae* to colonize baited pines (Borden et al. 1990).

Several earlier studies showed that (±)-*endo*-brevicomin can inhibit *D. frontalis* from responding to attractant-baited traps or landing on host trees (Vité and Renwick 1971; Payne et al. 1978; Richerson and Payne 1979; Salom et al. 1992). These findings prompted the conclusion that *endo*-brevicomin functioned as an antiaggregation pheromone for this species (Payne 1980; Skillen et al. 1997). However, after their discovery of the opposing activities of the enantiomers of *endo*-brevicomin, Vité et al. (1985) theorized that the inhibitory activity of the racemate was because of the antagonistic activity of the (−)-enantiomer overriding the attractive activity of the (+)-enantiomer.

We propose that male *D. frontalis* accelerate the aggregation of conspecifics to trees undergoing attack by releasing (+)-*endo*-brevicomin that acts synergistically with frontalin from females and host resin terpenes. The potential fitness benefits to pheromone-producing males are enhanced survival for both themselves and their offspring, as attracting greater numbers of attacking conspecifics should help assure rapid and complete disruption of host defenses. Alternately, males and females responding to hosts releasing (+)-*endo*-brevicomin, frontalin, and host terpenes (hosts infested with at least some beetle pairs) may avoid the high risks of mortality encountered by pioneer beetles that are first to initiate attacks on a particular tree (Pureswaran et al. 2006). Our data also indicate that the attractant currently used for monitoring populations of *D. frontalis* with traps (frontalin and turpentine; Skillen et al. 1997) is incomplete and that the addition of (+)-*endo*-brevicomin may enhance detection of low-density beetle populations. Additionally, this novel, enhanced-potency bait may prove efficacious in trap-out and other attractant-based management strategies for *D. frontalis.*

Acknowledgments We thank Brad Hoosier, Erich Vallery, Tessa Bauman, Chris Young, Jolie Mahfouz, James McDonald, and Alicia Niño for technical assistance; Drs. K. Fuhshuku and Y. Masuda of RIKEN, Japan, for help with the synthesis of (+)-*endo*-brevicomin; Mark Dalusky for providing us with *D. frontalis* from Alabama; Lee Dunnam, Homochitto National Forest, Mississippi, for assistance and cooperation in field studies; Larry Roton for useful insights that greatly influenced our thinking on this topic; Rhonda Tam and Jessica Norris for administrative assistance; Steve Clarke, Jim Meeker, and John Reeve for critical readings of earlier versions of this manuscript; and John Borden for extensive editing of the final text.

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behavior associated with feeding by larvae, much less is known about the mechanisms and ecological significance of experience-induced changes in host selection and oviposition behavior in adults (Papaj and Prokopy 1989; Schoonhoven et al. 2005). For example, oviposition on nonhost plants by various insects has been reported in both laboratory (Firempong and Zalucki 1990; Nylin et al. 2000; Solarz and Newman 2001) and field trials (Ampong-Nyarko et al. 1994; Prokopy et al. 1996), but little is known about the proximate mechanisms of this phenomenon and, consequently, sensory confusion or oviposition "mistakes" are often assumed (Larsson and Ekbom 1995). More recently, prior experience of nonhost plants by females has been found to induce oviposition on nonhost plants (Zhang and Liu 2006).

Olfactory cues are known to be central to host selection behavior in phytophagous insects (De Moreas et al. 2001; Schoonhoven et al. 2005). Here, we use laboratory experiments to examine whether early adult experience with nonhost plant odors can alter host selection behavior of the diamondback moth, *Plutella xylostella*, a specialist herbivore of cruciferous plants, so as to accept a nonhost plant for oviposition. In previous experiments with this insect, we moved mature larvae from host plants to pupate and emerge on nonhost plants. Thus, females with experience on nonhost plants were unavoidably associated with partial food restriction at the mature larval stage (Zhang and Liu 2006). Such food restriction may occur in nature when mature larvae facing food shortage are driven to move around and pupate on nonhost plants. Therefore, we examined the effect of larval food restriction on adult olfactory responses and oviposition preference. Our study demonstrates, for the first time, that olfactory experience during early adulthood alone plays a major role in the experience-induced changes of oviposition responses to nonhost plants. Insects that had experienced volatiles of a nonhost plant were attracted by its volatiles and were more likely to accept it for oviposition. Also, we show for the first time that larval starvation does not affect oviposition preference toward host and nonhost plants.

Methods and Materials

Insects and Plants *P. xylostella* was originally collected from a cabbage farm in a suburb of Hangzhou, China in October 2004 and maintained on common cabbage, *Brassica oleracea* L. var. *capitata*, cultivar Jing-feng No. 1, in a temperature-controlled room at 26±1°C, 60–70% RH with a 14L:10D photoperiod. The culture was replenished once a year with field-collected material and maintained for 10–15 generations when used in the experiments. The host plant Chinese cabbage, *Brassica campestris* L. ssp. *pekinensis*, cultivar Zao-shu No. 5, and a nonhost plant pea, *Pisum sativum* L. var. *arvense*, cultivar Zhongwan No. 2, were used in the olfactory and oviposition tests. All experiments utilized potted Chinese cabbage at the 5–6 fully expanded true leaf stage and potted pea with 20–25 fully expanded leaves grown in greenhouses under ambient temperature, humidity, and natural lighting. In olfactometer and oviposition choice tests, care was taken to use host plants and nonhost plants that had similar leaf areas.

Performance on Chinese Cabbage and Pea Plants: Survival, Development, and Body Size Four hundred *P. xylostella* eggs, deposited on plastic sheets, were taken from the *P. xylostella* culture. The eggs were randomly divided into two cohorts of 100 and 300, and the two cohorts of eggs were placed onto 10 plants of Chinese cabbage and 30 plants of pea (10 eggs on each plant), respectively. The two cohorts were reared at 26±1°C, 60–70% RH, and a 14L:10D photoperiod. Daily observations were made on survival, development, and

feeding until pupation. Pupae were collected within 24 hr of pupation and weighed individually to the nearest 0.1 mg (with a Mettler-Toledo AE200 electronic balance) as an index of body size. Pupae were placed individually in 2×8-cm glass tubes covered with a cotton plug until adult emergence. We recorded the following variables: (1) survival of eggs, larvae, and pupae, (2) development duration from egg to pupation, and (3) pupal weight.

Fecundity and Longevity Fecundity and longevity were observed by using detached leaves of Chinese cabbage as an oviposition substrate in a small rearing cage. The cage consisted of a clear, cylindrical, and ventilated plastic container (14.5-cm high, with a 9-cm-diameter top and 13-cm-diameter base). A cabbage leaf was fixed in the cage by inserting its petiole through a hole in the center of the base. The cage was placed onto a 500-ml glass bottle containing tap water, and the petiole of the cabbage leaf was inserted in the water to maintain freshness.

Twenty replicates were conducted for the cohort reared on Chinese cabbage. For the cohort reared on pea, no observation could be made on fecundity as only two male adults emerged. For each replicate, one female and one male (<24 hr postemergence) were placed into a rearing cage for mating and oviposition (15% honey–water solution provided as food). Eggs deposited on the leaf and cage walls were counted each morning, and adults were transferred to a new leaf in a clean cage until death. We assessed two variables: (1) total number of eggs laid and (2) longevity of adults.

Experience Treatments and Olfactory/Oviposition Preference: Experience Treatments To investigate the effects of early adult experience with pea volatiles or larval food restriction, or both, on the olfactory/oviposition preference of *P. xylostella* females, we used the following four treatments for the test insects.

(1) No experience and no food restriction. A batch of ~600 *P. xylostella* eggs were reared on four large plants of common cabbages at the precupping stage in a temperature-controlled room at 26±1°C, 60–70% RH, and a 14L:10D photoperiod. Each plant had 16–20 full-grown leaves, and the four plants provided more than enough food for development of the cohort from hatching to pupation. On the 13th day, approximately 500 pupae were obtained from the cohort. In an attempt to remove all external chemical cues from the larval environment, the pupae were taken out of their cocoons and thoroughly washed in demineralized water using the procedure described by Liu and Liu (2006b). After washing, pupae were placed into a sterilized cage (50×50×50 cm in size) for adults to emerge. Until used in olfactory/oviposition preference tests at 2–3 d postemergence, adults were not exposed to any plant odors.
(2) Larval food restriction. Insects were reared the same way as in (1), except that mature fourth instars were moved manually onto plastic sheets for them to pupate. Pupae were washed using the same procedure as above. After washing, pupae were placed into a sterilized cage (50×50×50 cm in size) for adults to emerge. Adults were deprived of any plant odors as in (1) before being used in olfactory/oviposition preference tests at 2–3 d postemergence.
(3) Pea odor experience. Insects were reared, and pupae were washed the same way as in (1). After washing, pupae were placed into a sterilized cage (40×40×40 cm in size) for adults to emerge.

A wind tunnel (Fig. 1) was set up in a separate temperature-controlled room at 25±2°C. Five potted pea plants were placed into the chamber in front of the fan, and the *P. xylostella* pupae, from which adults were to emerge in a day, were placed into the next chamber

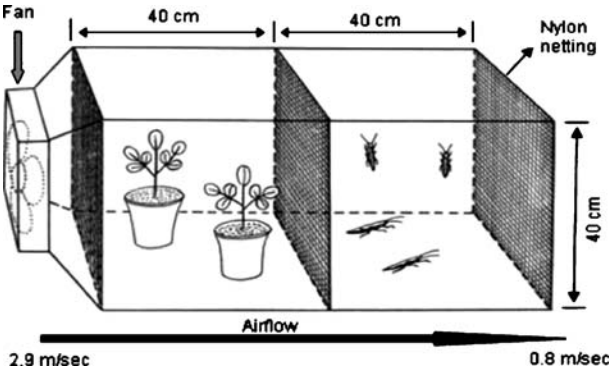


Fig. 1 Diagram showing how moths were exposed to odors of nonhost pea plants in a wind tunnel

(Fig. 1). The wind speed at the inlet of the tunnel was approximately 2.9 m/sec and 0.8 m/sec at the outlet. Consistent airflow was maintained after the placement of pupae into the tunnel. Adults were exposed to the pea volatiles during emergence and for 2–3 d after emergence, before they were used in olfactory/oviposition preference tests.

(4) Pea plant experience. Insects were reared on common cabbage from egg to late fourth instar as in (1) above, and then the mature larvae were moved manually onto pea plants to force them to pupate on the nonhost. Insects were left to emerge naturally on the pea plants. Adults were enclosed with the pea plants in a cage until used in olfactory/oviposition preference tests at 2–3 d postemergence.

Thirty pupae were randomly taken from each of four treatments and weighed individually as an index of body size. Adults were always provided with 15% honey–water as food upon emergence.

Olfactory Responses The responses of *P. xylostella* females to volatiles of Chinese cabbage and/or pea plants were assessed with a Y-tube olfactometer. The transparent glass Y-tube olfactometer had a 20-cm stem and two 20-cm arms at a 75° angle, and its internal diameter measured 4 cm (Fig. 2). At the middle of each arm, there was a glass funnel (diameter of port = 8 mm) pasted on the inner wall. Just after this port, there was a 25-mm vertical male ground-glass connector for a 100-ml "moth-trapping" bulb. A stainless steel mesh screen was placed just after the connector and served to block the passage of the moths and force them to move upward into the moth-trapping bulb (Fig. 2). Each arm ended with a 25-mm female ground-glass connector, and was then connected to separate glass containers holding the odor sources. Air was filtered through activated charcoal, humidified, and split into two air streams that fed through the glass containers to the olfactometer at a rate of 200 ml/min in each arm. All bioassays were conducted in a temperature-controlled room at 25°C, illuminated only by a 15 W red light, between 19:00–23:00 h when the moths were expected to be most active.

For each replicate, a group of four female moths were released at the down-wind end of the Y-tube stem and left in the olfactometer to respond for 15 min. When a moth walked up to the upwind end of an olfactometer arm, either within 4 cm of the blocking screen or into the corresponding moth-trapping bulb at the end of 15 min, it was recorded as making a choice for the odor of that arm. When a moth did not reach the end of either arm within 15 min, it was recorded as "no response". Each moth was used only once. After testing five groups of moths, the connections of the odor sources to the olfactometer arms were

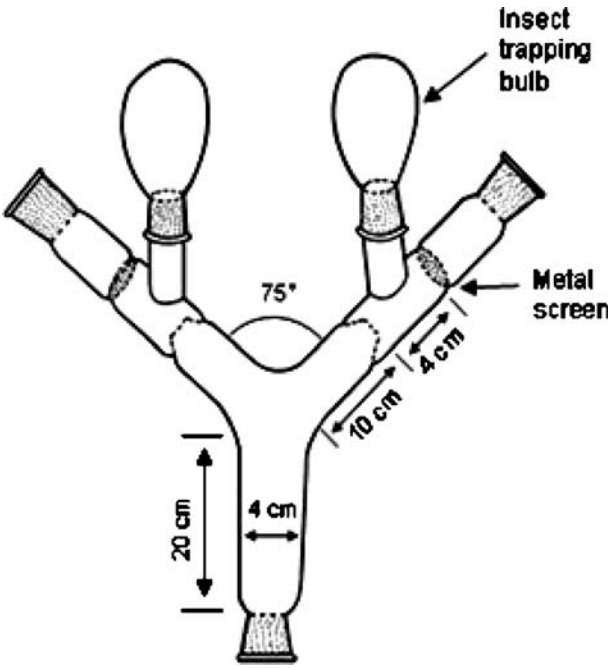


Fig. 2 An overview of the Y-tube olfactometer used in choice tests. The insect-trapping bulb enabled moths making a choice to be more easily identified when testing more than 1 moth in the olfactometer

exchanged to remove any asymmetrical bias in the set-up and the test plants were replaced by new ones of the corresponding categories. The olfactometer tube and glass container were thoroughly washed with alcohol at the end of the observations for a given treatment, and dried overnight in an oven at 200°C.

We first tested the responses of control moths from the culture to the apparatus with just clean air flowing down each arm. Mated females at 2–3 d postemergence were used, and 64 moths were tested to observe the background rate of "no response" as well as any potential bias in arms.

The responses of moths in the "no experience" and "pea plant experience" treatments were tested to odors of each of the following three choices: Chinese cabbage vs. air, pea vs. air, and Chinese cabbage vs. pea. Moths in both "larval food restriction" and "pea odor experience" treatments were tested only to odors of the dual-choice of Chinese cabbage vs. pea. Twenty groups of four female moths each (i.e., 80 moths in total) were assessed for each combination of experience treatment and volatile choice.

Oviposition Preference Oviposition by *P. xylostella* females from each of the four experience treatments was tested in two no-choice tests and/or one choice test:

(a) No-choice test with host plant. One Chinese cabbage plant was placed into the center of a ventilated cage constructed with a wire metal frame covered on all sides with black plastic sheets. The cage had an internal volume of 40×40×40 cm with two 15×15 cm screened windows on the front and backsides for ventilation. At 19:00, four females were released into each cage (no food was provided). After 12 hr in darkness at 26°C, moths were removed, and all eggs deposited on each plant, pot, and the inner surface of the cage, were counted.

(b) No-choice test with nonhost plant. The same procedure as in (a) was used, except that pea was used as the test plant.
(c) Choice test with host and nonhost plants. One Chinese cabbage and one pea were placed in diagonally opposite corners of the same cage, so that the foliage of the two plants was approximately 20 cm apart at the closest point. At 19:00, four females were released into the center of each cage. After 12 hr in darkness at 26°C, the moths were removed and the eggs deposited on each of the two plants, the pots, and inner surface of the cage, were counted.

Ten replicates (four moths per replicate) were conducted for each experience treatment.

Statistical Analysis All proportional data were transformed by arcsine square root for analysis and were backtransformed to percentages for presentation. For comparing performance of *P. xylostella* on cabbage and pea, percentages or mean values between the two plants were analyzed with Student *t* test. For the olfactometer bioassays, a replicated *G* test of goodness-of-fit was used to analyze the frequency distributions of the choices made by females in each treatment, with the null hypothesis of no preference, and a test of independence in two-way tables was used to compare frequency distributions between different treatments (Sokal and Rohlf 1995). The mean numbers of eggs laid by females among the eight plants × experience treatments were analyzed by one-way ANOVA, and means were compared pairwise by using the LSD procedure. The mean proportions of eggs deposited on cabbage or pea plants in the no-choice tests between females from "no experience" and "pea plant experience" treatments were analyzed with Student *t* test. The mean proportions of eggs deposited on pea plants in the dual-choice tests among females from the four experience treatments were analyzed with one-way ANOVA, and means were compared pairwise by using the LSD procedure. The statistical software, BIOMstat (version 3.30 q, Applied Biostatistics, Inc., NY, USA) was used for the statistical analysis.

Results

Performance on Chinese Cabbage and Pea Plants The two cohorts of eggs for tests on Chinese cabbage and pea had similar and high percentages of hatching (Table 1). Little feeding by larvae occurred on pea compared to that on Chinese cabbage. By the 12th day after hatching, most larvae on Chinese cabbage developed normally to pupation, whereas 266 of the 280 larvae on pea died in the first or second instar, and the remaining 14 were still small. Successful survival from hatching to pupation was low on pea compared to that on Chinese cabbage (Table 1). The development time from egg hatch to pupation on pea was twice as long as that on Chinese cabbage, whereas the weight of pupae from pea was only half of that from Chinese cabbage (Table 1). Females reared from Chinese cabbage laid on average 345 eggs, whereas no females were obtained from pea. For this reason, fecundity could not be assessed on this plant (Table 1).

Olfactory and Oviposition Preference The average weights of pupae differed among the four experience treatments ($F=12.96$, $df=3$, $P<0.001$). Compared to the pupal weight in the "no experience" treatment (6.8 mg), the pupal weights in both "larval food restriction" (5.4 mg) and "pea plant experience" (5.7 mg) treatments were significantly lower (larval food restriction: $P<0.001$; pea plant experience: $P<0.001$), but that in the "pea odor experience" treatment (6.4 mg) was not ($P=0.09$).

Table 1 Performance of *Plutella xylostella* reared on Chinese cabbage and pea plants

Parameters	Plants	
	Chinese Cabbage	Pea
Total number of eggs at start of test	100	300
% Survival of eggs	95.0 a	93.3 a[a]
% Survival from hatching to pupation	81.0 a	1.1 b
Development time (days) from hatching to pupation	10.2±0.1 b	21.0±0.1 a
Pupal weight (mg)	5.9±0.1 a	2.8±0.1 b
% survival of pupae	97.4 a	66.7 b
Number of eggs laid per female	345.3±13.3	–[b]
Adult longevity (days)	9.7±0.3 a	7.3±0.5 b

[a] Percentages or means ± SE on the same row followed by the same letter do not differ significantly ($P<0.05$).

[b] As only two male adults emerged on pea, no observation on fecundity could be conducted.

Olfactory Responses In the tests with 64 control moths, 21 (32.8%) exhibited "no response," while 24 (37.5%) and 19 (29.7%) responded to the two arms, respectively. The results indicated no bias between the arms ($G=0.576$, $df=1$, $P=0.45$).

A higher proportion (70%) of moths in the "no experience" treatment made their choice toward Chinese cabbage, when provided with a choice between volatiles emanating from Chinese cabbage and air. In contrast, the proportion of moths in the "pea plant experience" treatment that chose Chinese cabbage decreased to 45%, resulting in similar responses to Chinese cabbage and air. The relative proportions of moths making their choice between Chinese cabbage and air differed significantly between the two treatments (Fig. 3a).

A lower proportion (31%) of moths in the "no experience" treatment made their choice toward pea rather than air, when provided with a choice between volatiles emanating from pea and air. Remarkably, the proportion of moths choosing pea increased to 62% in the "pea plant experience" treatment, resulting in a significant preference for pea. The relative proportions of moths making their choice between pea and air differed significantly between the two treatments (Fig. 3b).

When provided with a choice between volatiles of Chinese cabbage and those of pea, a higher proportion (64%) of moths made their choice toward Chinese cabbage in the "no experience" treatment. The proportion of moths choosing Chinese cabbage decreased to 55% in the "larval food restriction" treatment, but the relative proportions of moths making their choice toward the two plants did not differ between the two treatments (Fig. 3c). In contrast, the proportion of moths choosing Chinese cabbage decreased to 32 and 40% in the "pea odor experience" and "pea plant experience" treatments, whereas the proportion of moths choosing pea correspondingly increased in the two treatments (Fig. 3c). The relative proportions of moths making their choice toward the two plants did not differ between the "pea odor experience" and "pea plant experience" treatments, but the relative proportions of these two treatments differed from that of the "no experience" treatment (Fig. 3c).

Number of Eggs Laid The number of eggs laid on Chinese cabbage by females in "no experience" treatment can be regarded as the normal level of oviposition by the test insects. When provided with Chinese cabbage, the females in the "pea plant experience" treatment laid significantly fewer eggs compared to the normal level. When provided with pea, females in the "no experience" treatment laid only one third of the eggs of the normal level,

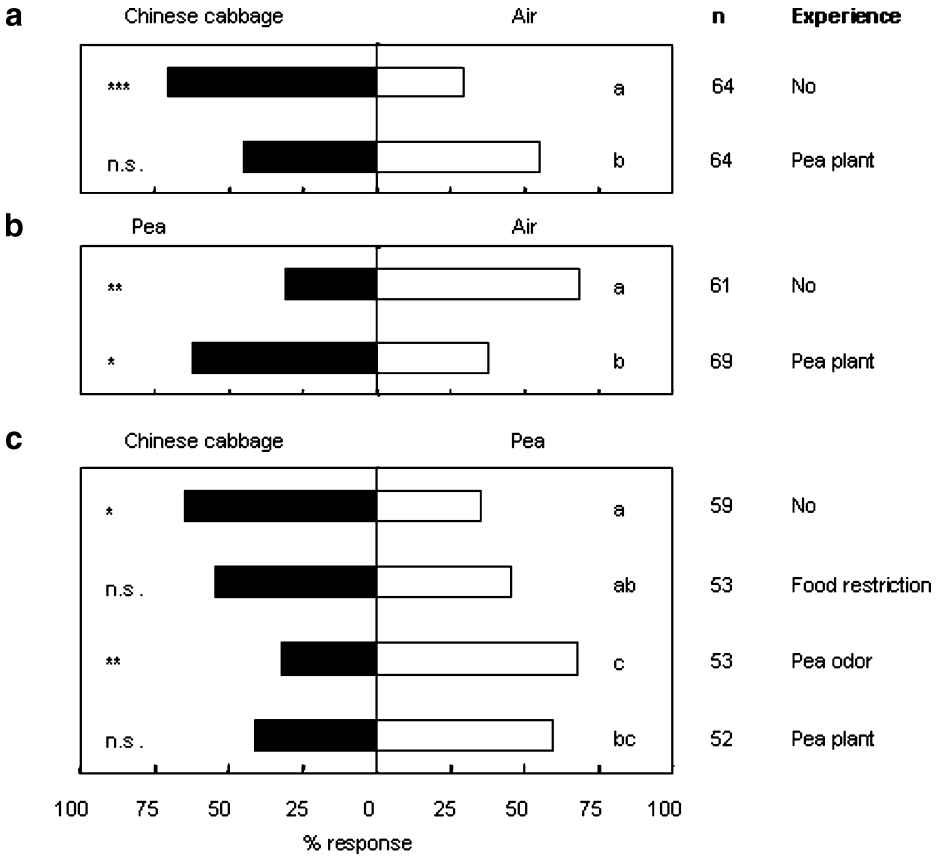


Fig. 3 Effect of early adult experience of pea in the form of odor or whole plant as well as effect of larval food restriction on responses of *Plutella xylostella* females to volatiles of "Chinese cabbage vs. air" (**a**), "pea vs. air" (**b**), and "Chinese cabbage vs. pea" (**c**) in a Y-tube olfactometer. The number of females (*N*) that made a choice in a treatment is given on the right side of the bars; asterisks on the left indicate significant differences within a choice test (**P*<0.05, ***P*<0.01, ****P*<0.001, n.s. = not significant). Within figures **a**, **b**, or **c**, the bars of different experience treatments followed by the same letter on the right indicate no significant difference (*P*<0.05) between them

and those in the "pea plant experience" treatment also laid fewer eggs than the normal level (Table 2). When provided with Chinese cabbage and pea, females in the "no experience" and "pea odor experience" treatments laid similar numbers of eggs compared to the normal level; females in the "larval food restriction" and the "pea plant experience" treatment appeared to lay fewer eggs than the normal level. However, the difference between either of the two treatments and the normal level was not significant due to the large variation among replicates (Table 2).

Oviposition Preference In no-choice tests that used Chinese cabbage, females of the "no experience" and "pea plant experience" treatments laid on average 96 and 99% of their eggs on the host plants, with the remainder on pots and cage surface. The proportions of eggs laid on cabbage did not differ between the two treatments (Table 3).

In no-choice tests with pea, the proportion of eggs laid on pea by females of the "pea plant experience" treatment was significantly higher than that of the "no experience"

Table 2 Mean number of eggs laid on Chinese cabbage and/or pea by *Plutella xylostella* females that experienced larval food restriction, pea odor, or pea plants

Plant	Experience Treatments			
	No Experience	Larval Food Restriction	Pea Odor	Pea Plant
Cabbage only	102.4±14.4 ab[a]	–	–	67.3±6.0 cd
Pea only	28.3±4.2 e	–	–	53.7±4.6 d
Cabbage and pea	98.8±13.0 ab	86.0±11.0 abc	111.4±14.0 a	78.2±10.3 bcd

[a] Ten replicates were conducted for each of the eight plant×experience treatments, means ± SE in the table followed by the same letter do not differ significantly ($P<0.05$).

treatment, although females in both treatments laid the majority of their eggs on pots and cage surface (Table 3).

In choice tests with Chinese cabbage and pea, females in each of the four treatments laid >90% of their eggs on the two plants, with the remainder on pots and cage surface (not considered in the data analysis). Although females in each of the four experience treatments laid nearly all, or the majority, of their eggs on Chinese cabbage, the proportions of eggs laid on pea differed among the four treatments ($F=15.7$, $df=3$, $P<0.001$). Compared to the proportion of eggs laid on pea by females of the "no experience" treatment, the proportion was higher in the "pea odor experience" ($P<0.001$) and "pea plant experience" treatment ($P<0.001$), but that of the "larval food restriction" treatment was not (Fig. 4). Moreover, the proportions in the "pea odor experience" and "pea plant experience" treatments were similar ($P=0.88$; Fig. 4).

Discussion

Survival of *P. xylostella* from egg to adult on the pea cultivar tested was <1%, and the few resulting adults were much smaller, as indicated by their reduced pupal weight, than those that fed on Chinese cabbage (Table 1). Our cohort on pea only produced two adult males, so it is not clear if *P. xylostella* developing on this pea cultivar are capable of producing eggs, a requirement for a plant to be considered a host (Ward 1988). In view of the very low survival of the test cohort on pea and the lack of records of *P. xylostella* larval populations on any pea plants in the field from China, we regard the pea cultivar used in this study as a nonhost plant of the local *P. xylostella* population.

Naïve *P. xylostella* females showed an aversion to volatiles emanating from pea (Fig. 3b), and rarely accepted pea for oviposition (Table 3 and Fig. 4). However, when newly emerged females were given prior experience with pea plants, they showed a

Table 3 Percentages of eggs laid on chinese cabbage or pea plants in no-choice tests by *Plutella xylostella* females with or without prior experience of pea plants

Experience Treatment	Plants in No-Choice Tests[a]	
	Cabbage	Pea
No experience	96.2±1.3 a	3.3 ± 1.5 a
Pea plant experience	98.9±0.6 a	20.3 ± 8.3 b

[a] Ten replicates were conducted for each of the four experience×plant treatments, means ± SE in the same column followed by the same letter do not differ significantly ($P<0.05$).

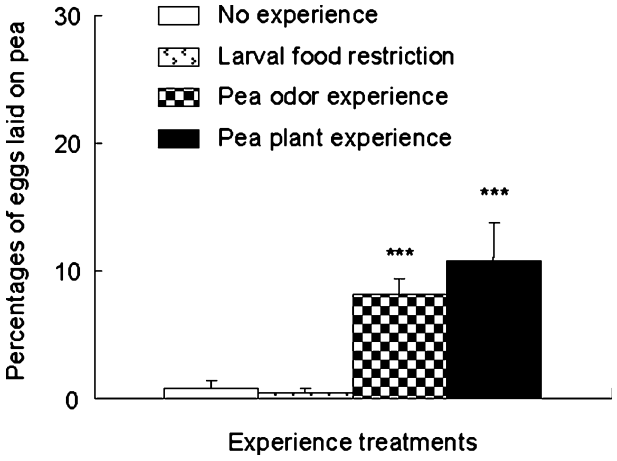


Fig. 4 Percentages of eggs laid on pea plants by *Plutella xylostella* females with various prior experiences in dual-choice tests with Chinese cabbage and pea. Ten replicates were conducted for each of the four experience treatments. Error bars indicate standard error of means, and an asterisk above a column indicates significant difference between that treatment and the "no experience" treatment (***$P<0.001$)

preference for volatiles of pea (Fig. 3b), and increased their acceptance of pea for oviposition in both no-choice and choice conditions (Table 3 and Fig. 4). More remarkably, females that were given an experience of pea volatiles alone, without physical contact of the plants, similarly increased their preference for volatiles of pea and their acceptance of pea plants for oviposition (Figs. 3c and 4). Females in the "larval food restriction" treatment experienced a level of food shortage during the mature larval stage similar to that of the females in the "pea plant experience" treatment, and their olfactory responses and oviposition preference were unaffected (Figs. 3c and 4). Thus, it seems likely that the oviposition behavior of females in the "pea plant experience" treatment was little affected by larval food restriction, and their altered oviposition preference (Fig. 4) was due mainly to olfactory experience. Previous studies have shown that experience of nonhost plants (Zhang and Liu 2006) or nonhost plant chemicals (Liu et al. 2005; Liu and Liu 2006a) by *P. xylostella* can change subsequent oviposition behavior, but these studies could not distinguish between contact experience of nonhost semiochemicals and olfactory experience of volatiles.

In our experiments, females in the "no experience" and "larval food restriction" treatments exhibited similar olfactory responses and nearly identical oviposition preference toward Chinese cabbage and pea plants (Figs. 3c and 4), although the females from "larval food restriction" had a lower pupal weight as well as a lower number of eggs laid on the first day when plants were offered (Table 2). Thus, partial food restriction at the mature larval stage reduced fitness, but did not alter oviposition behavior of the ensuing females. The unchanged oviposition behavior suggests that partial food restriction did not affect oviposition motivation of the ensuing adults, or that the treatment affected oviposition motivation, but oviposition motivation did not alter oviposition preference (Monks and Kelly 2003). To our knowledge, the effect of larval food restriction on oviposition preference among plants has not been examined for any other insects, although many studies have been conducted to investigate the effect of larval environment on oviposition behavior through retention of learning. These studies generally demonstrate that preimaginal conditioning does not affect oviposition preference in holemetabolous insects (Papaj and Prokopy 1989; Rojas and Wyatt 1999; Liu and Liu 2006b).

The modification of host selection behavior by experience is a widespread phenomenon in phytophagous insects (Papaj and Prokopy 1989; Schoonhoven et al. 2005). Although empirical evidence for such learning-associated behavior has been collected mostly from

larvae (Jermy 1987; Schoonhoven et al. 2005), documented cases for adults have been increasing (Cunningham et al. 1998a, b; Rojas and Wyatt 1999; Held et al. 2001; Cunningham et al. 2004; Liu et al. 2005). These case studies of changes in behavior due to olfactory experience, especially changes in oviposition preference, indicate that oviposition on nonhost plants due to olfactory learning, as exhibited by *P. xylostella*, may occur in many phytophagous insects, especially for night-flying insects that use mainly plant volatiles to locate host plants for oviposition, with visual cues playing a minor or negligible role in host plant finding (Schoonhoven et al. 2005; Couty et al. 2006).

Females in the "pea odor experience" and "pea plant experience" treatments were more attracted by pea volatiles than by volatiles of Chinese cabbage (Fig. 3c), yet they deposited only about 10% of their eggs on pea plants in the dual-choice oviposition preference tests with these two plants (Fig. 4). The contrary responses between olfaction and oviposition seemed to indicate that physical features and/or contact chemicals of pea deterred acceptance and oviposition by *P. xylostella*.

In nature, *P. xylostella* usually pupate on host plants, and females experience first the semiochemicals of the host plants on which they emerge. Females have a strong innate preference for volatiles of cruciferous plants (Fig. 3, Liu et al. 2005; Couty et al. 2006), and olfactory learning further increases their preference for the volatiles they first experience (Liu and Liu 2006a, b). Consequently, newly emerged females usually land and accept cruciferous host plants for oviposition, and the insect species runs little risk of laying eggs on nonhost plants.

A small proportion of naïve females oriented to pea odors (Fig. 3b), although this proportion could not be distinguished from females that may simply have been responding to air movement. Gupta and Thorsteinson (1960) found that a population of *P. xylostella* from Canada showed some olfactory response to pea plants. It is possible that there is a degree of preadaptation to this nonhost through shared common host signals, and that experience increases moth responsiveness to the nonhost plant, similar to the findings of Bengtsson et al. (2006). In our study, *P. xylostella* achieved very low survival on cultivar Zhongwan No. 2 of *Pisum sativum* L. var. *arvense*, and the same experimental population of this insect achieved virtually no survival on cultivar Qiwanyiji of the same variety of pea in a previous study (Zhang and Liu 2006). However, in a separate experiment, we found that this population of *P. xylostella* achieved 6% survival to adulthood on cultivar Zhongwan No. 5 of *Pisum sativum* L. var. *arvense*, and the ensuing females laid on average 107 eggs on Chinese cabbage (unpublished data). Gupta and Thorsteinson (1960) also demonstrated low levels of survival on pea for *P. xylostella*. It appears that different varieties and cultivars of pea vary in suitability to performance of *P. xylostella*, and it is possible that geographic populations of *P. xylostella* vary in their physiological adaptation to utilize pea plants. Larval performance on novel host plants may show a higher variability than on normal host plants, and the heritability for growth performance may also be higher on novel host plants (Ueno et al. 2001). Thus, if the behavioral barrier for adults is overcome and a large number of eggs are laid on novel plants, survival of the immature stages may be selected for (Löhr and Gathu 2002). Subsequently, if some offspring show an increased acceptance of novel host plants, as a result of either a conditioned or inherited behavior (Hora et al. 2005; Zhang and Liu 2006), further oviposition on novel plants could act to initiate a host range expansion.

Such experience induced oviposition activities on novel plants by phytophagous insects may be more likely to happen in crop ecosystems. For example, in many crop systems novel crop species are cultivated as monocultures over large areas, and, consequently, the ovipositing females repeatedly encounter the novel plants in host foraging. In more

diversified crop systems, plots of host crops are surrounded by plots of nonhost crops, and when host crops are suddenly harvested, individuals of the pest insects, including mature larvae and adults, left without food are forced to move onto the nearby nonhost crops. In Kenya, a field *P. xylostella* population on pea crops was first found when the population density on the usual host plants in the area was very high (Löhr and Gathu 2002).

Insect–plant associations show a general pattern of predominance of specialists over generalists and a strong taxonomic conservation in host plant use (Lopez-Vaamonde et al. 2003; Schoonhoven et al. 2005). However, recent studies on the directionality of host range evolution in phytophagous insects suggest that host range evolution is a highly dynamic process and seems characterized by repeated phases of contraction and expansion (Janz et al. 2001; Termonia et al. 2001; Nosil 2002; Lopez-Vaamonde et al. 2003; Janz et al. 2006). Many phytophagous insects are capable of including new plant species within their diets. Cases of host range expansion by phytophagous insects have been observed to include novel plant species from the same genus or family (Feder et al. 1990; Berenbaum and Zangerl 1991; Carroll and Boyd 1992; Fox et al. 1997; Louda et al. 1997; Solarz and Newman 2001) or from different families (Bowers et al. 1992; Singer et al. 1993; Camara 1997). For phytophagous insects with relatively immobile larvae, such as many Lepidoptera, colonization of a potential host plant may be precluded by the failure of females to recognize the potential host as a suitable oviposition site. Larvae can often feed and survive on plants that are not usually accepted as oviposition substrates by adult females (Thomas et al., 1987; Ueno et al. 2001; Schoonhoven et al. 2005). Thus, behavioral adaptations, such as changes of host selection behavior induced by olfactory experience, may be the primary mechanism driving novel host associations, with physiological adaptations a secondary result of behavioral changes (Wcislo 1989; Gassmann et al. 2006).

In many cases of host range expansion, native insects have incorporated exotic plants into their diet (Thomas et al. 1987; Feder et al. 1990; Carroll and Boyd 1992; Fox et al. 1997; Solarz and Newman 2001; Carroll et al. 2005), or exotic insects have incorporated native plants into their diet (Berenbaum and Zangerl 1991; Bowers et al. 1992; Louda et al. 1997). In fact, the majority of phytophagous insects accumulated by introduced plants, such as cacao and sugarcane, are recruited from the native, local faunas in the regions of introduction (Strong 1979). As biological invasions are occurring at a higher rate with each passing year, chances for new associations between insects and plants are increasing. New introductions of exotic insects, as well as the environmental changes of decreasing availability of ancestral hosts and increasing availability of exotic hosts, will present more opportunities for host range expansion to occur (Bowers et al. 1992; Singer et al. 1993). Consequently, exotic insects may feed on novel, native plants and so speed up the success of invasion, whereas native insects may feed on novel, exotic plants and thus contribute to the resistance of community to biological invasion.

Acknowledgements This study was funded by the National Natural Science Foundation of China (Project no. 30571225) and the National Basic Research and Development Program of China (2002CB111403). We thank Paul Cunningham, John Moores University, UK and Mike Furlong, University of Queensland, Australia, for helpful comments on the manuscript.

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Introduction

Although plants accumulate hundreds of different defensive compounds that prevent herbivore consumption, herbivores themselves have devised enzymatic means that detoxify plant defensive metabolites. Recently, two reports have demonstrated the detoxification of glucosinolates by insects (Ratzka et al. 2002; Wittstock et al. 2004). Glucosinolates are a group of *S*-β-glucosides found in members of the plant order Capparales (Halkier and Gershenzon 2006) that are present in crop plants such as broccoli, cabbage, and rocket salad, as well as in wild crucifers such as desert cabbage (*Schouwia purpurea*) and stinkweed (*Thlaspi arvense*). They are stored in the vacuole of the plant cell and are hydrolyzed by the enzyme myrosinase upon disruption of the cell by insect feeding or mechanical disturbance. Myrosinase, a thioglucosidase, cleaves glucose from the core glucosinolate structure, yielding an unstable amino acid-derived aglucone that rapidly rearranges to form a variety of products including isothiocyanates, thiocyanates, epithionitriles, and nitriles. This so-called "mustard oil bomb" (Matile 1980) releases volatile products of glucosinolate hydrolysis that are often toxic to insects (Wittstock et al. 2003). Some insects, however, have overcome the toxicity of glucosinolates and incorporate glucosinolate-containing plants into their diets. Specialist herbivores, such as larvae of the diamondback moth (*Plutella xylostella*), larvae of the cabbage white butterfly (*Pieris rapae*), the cabbage stem flea beetle (*Psylliodes chrysocephala*), the cabbage aphid (*Brevicoryne brassicae*), and the crucifer flea beetle (*Phyllotreta cruciferae*), feed exclusively on glucosinolate-containing plants, whereas generalist herbivores including slugs and snails, the aphid *Myzus persicae*, and the desert locust *Schistocerca gregaria* feed on a larger variety of plants, only some of which contain glucosinolates.

Two different enzymatic mechanisms have been described for the detoxification of glucosinolates by specialist herbivores. Diamondback moth larvae possess an enzyme, glucosinolate sulfatase (GSS), which cleaves the sulfate group yielding desulfoglucosinolates (Ratzka et al. 2002). Because the plant enzyme myrosinase catalyzes glucose hydrolysis only from intact glucosinolates and not from desulfoglucosinolates, no toxic glucosinolate hydrolysis products are formed in the presence of GSS. In larvae of *Pieris rapae*, however, a different method is employed by the insect for circumventing the toxic effects of glucosinolates in its diet (Wittstock et al. 2004). *P. rapae* larvae possess a "nitrile-specifying protein" (NSP) in their gut. NSP prevents the formation of isothiocyanates during glucosinolate hydrolysis, instead directing the aglucone of the glucosinolate to form a nitrile. Nitriles are less toxic than isothiocyanates, and the presence of this enzyme allows *P. rapae* larvae to feed extensively on plants containing glucosinolates.

Generalist herbivores that occasionally feed on glucosinolate-containing plants must also be able to avoid the toxic effects of these plant compounds, but little is known about the mechanisms involved. The desert locust, *Schistocerca gregaria*, is a generalist feeder that includes the annual crucifer *Schouwia purpurea* in its diet (Ghaout et al. 1991; Culmsee 2002). Although *S. purpurea* has a glucosinolate content tenfold higher than most cruciferous crop plants (Ghaout et al. 1991; Fahey et al. 2001), the desert locust is reported to eat the plant without any detrimental effects (El Sayed et al. 1996; Mainguet et al. 2000). Thus, this insect must be adept at circumventing glucosinolate toxicity. In this report, we show that desert locusts possess glucosinolate sulfatase activity in their gut, which alters the core structure of glucosinolates, preventing the release of toxic hydrolysis products by myrosinase. The enzyme is induced to higher levels when locusts feed on glucosinolate-containing plants. The ability of a generalist herbivore to modulate the level of detoxification machinery in response to the presence and absence of a toxin may reduce the physiological costs of detoxification.

Materials and Methods

Plants, Insects and Other Materials Seeds of *S. purpurea*, collected from wild plants in Mauritania, were a gift of Mohamed Lemine, FAO Locust Center, Nouakchott, Mauritania. Plants were germinated and grown in ordinary potting soil (Klasmann–Deilmann Tonsubstrat, Geeste–Gross Hesepe, Germany) in a greenhouse under a diurnal cycle of 14:10 h L:D at 22–26°C. Desert locusts (adult females unless otherwise indicated) were provided from a culture reared at Martin Luther University, Halle, Germany as described (Seidelmann et al. 2005) and were maintained in a growth chamber at 35°C during the day and 25°C during the night, 30% relative humidity, and a 12:12 h L:D cycle. In culture, before experimental feeding of *S. purpurea*, locusts were fed primarily with wheat grass and dry oatmeal with occasional supplements of Chinese cabbage and savoy cabbage. Most of the glucosinolate substrates used (indol-3-ylmethyl-, (*S*)-2-hydroxy-3-butenyl-, and 3-butenyl-glucosinolate) were isolated in the laboratory. However, sinigrin and all other chemicals were purchased from Sigma.

Glucosinolate and Glucosinolate Product Analysis Glucosinolates were extracted from *S. purpurea* leaves, stems, and flowers by using a modification of the method described in Brown et al. (2003). Fresh tissue (100–300 mg) was homogenized by a Polytron in 4 ml of 80% (aqueous) methanol containing 0.05–0.1 μmol *p*-hydroxybenzyl glucosinolate as an internal standard and 30 μmol $Pb(OAc)_2$ and 30 μmol $Ba(OAc)_2$ to precipitate protein and free sulfate ions. Samples were centrifuged at 4,000×*g* for 10 min, and the resulting supernatant was loaded onto an anion-exchange column (DEAE-Sephadex A-25) to bind intact glucosinolates. The column was washed sequentially with 67% (aqueous) methanol and 0.02 M Mes, pH 5.2, before being capped overnight with 50 μl of a *Helix pomatia* sulfatase solution. The resulting desulfated glucosinolates were eluted from the column with 60% (aqueous) methanol, and the eluent was evaporated to dryness under nitrogen at 50°C before being resuspended in water. To analyze both desulfoglucosinolates and intact glucosinolates from desert locust feces, the eluent obtained during sample loading on the anion exchange column and washing of the column was retained. This fraction, which would be expected to contain any desulfoglucosinolates present, was evaporated to dryness as described above and resuspended in water for high performance liquid chromatography (HPLC) analysis. The intact glucosinolates from feces that bound to the anion-exchange column were desulfated as already described.

Samples were separated by HPLC (Agilent HP1100 Series) fitted with a C-18 reversed-phase column (Supelcosil RP-18, 250×4.6 mm i.d., 5-μm particle size, Supelco, Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) using a water (Solvent A)-acetonitrile (Solvent B) gradient at a flow rate of 1 ml min^{-1} at ambient temperature. The 38-min run consisted of 1.5% B (1 min), 1.5–5.0% B (5 min), 5.0–7.0% B (2 min), 7.0–21.0% B (10 min), 21.0–29.0% B (5 min), 29.0–43.0% B (7 min), 43.0–100.0% B (0.5 min), a 2.5-min hold at 100.0% B, 100.0–1.5% B (0.1 min), and a 4.9-min hold at 1.5% B. Eluent was monitored at 229 nm. Desulfated glucosinolates were identified by comparison of their retention times and UV spectra to those of purified standards previously extracted in our laboratory (Reichelt et al. 2002; Brown et al. 2003). Compounds were quantified by A_{229nm} relative to the internal standard with response factors reported in the literature (Brown et al. 2003). To confirm the presence of desulfoglucosinolates in feces, extracts were incubated with *Caldocellum saccharolyticum* β-*O*-glucosidase, an enzyme shown to hydrolyze only desulfoglucosinolates and not intact glucosinolates (Wathelet et al. 2001).

Analysis of glucosinolate hydrolysis products originating from *S. purpurea* leaves, flowers, and stems,and in desert locust feces was performed as described in Lambrix et al. (2001).

Enzyme Extraction, Assay, and Characterization Insects were chilled at −20°C for 20 min before dissection. The entire gut was removed from the thorax and abdomen, rinsed in 20 mM Mops, pH 6.9, and homogenized via Polytron in 5 ml of the same buffer. The homogenate was centrifuged for 20 min at 48,000×*g*, and the supernatant was reserved for assay. The standard assay consisted of 10 mM of glucosinolate and 1–5 μg total protein in 100 μl of 20 mM Mops, pH 6.9, and was conducted at 25°C. The reaction was initiated by addition of the substrate glucosinolate and was stopped by the addition of 500 μl of methanol after 4 min. To separate intact glucosinolates from desulfoglucosinolates, 200 μl of a 10% (w/v) aqueous suspension of DEAE-Sephadex were added to the assay. DEAE-bound intact glucosinolates were removed from the suspension by filtration over glass wool. The eluate containing desulfoglucosinolates was prepared for HPLC as described above for glucosinolate extraction from plant tissues, except that samples were dissolved in 20 mM Mops, pH 6.9 containing 250 μg ml^{-1} myrosinase to hydrolyze any residual intact glucosinolates that did not bind to the DEAE.

The cell-free gut extract was assayed for arylsulfatase activity by combining 20 μg of total protein and 5 mM 4-nitrocatechol sulfate with 10 mM Tris-HCl, 100 mM KCl, and 5 mM ethylenediamine tetraacetic acid (EDTA), pH 6.3, in a total volume of 0.5 ml. After 60 min incubation at 25°C, the reaction was stopped by the addition of 0.5 ml of 5 N NaOH, and the absorbance of the resulting 4-nitrocatechol was measured at an absorbance of 515 nm. For inhibitor experiments, EDTA was omitted from the assay buffer. Protein levels were determined by using the Bio-Rad reagent with BSA as the standard.

Statistical Analysis Statistical analysis of intact glucosinolate and glucosinolate hydrolysis product variation among plant organs, as well as variation in glucosinolate sulfatase activity during locust development and under different feeding protocols, was accomplished either by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test or by Kruskal–Wallis one-way ANOVA followed by Dunn's test as indicated in the figure legends. The software used was SigmaStat 3.5, and significant differences were determined at $P<0.05$.

Results

Glucosinolate Composition of S. purpurea To determine the glucosinolate composition that desert locusts are exposed to when feeding on *S. purpurea*, a typical component of its diet, tissues of this species were extracted, and the isolated glucosinolates were analyzed by HPLC. The glucosinolate profiles of leaves, flowers, and stems (Fig. 1) are similar, dominated by 3-butenyl glucosinolate (64–92% of total glucosinolates), with significant amounts of (*S*)-2-hydroxy-3-butenyl glucosinolate (5–22%) and indol-3-ylmethyl glucosinolate (2–19%). In all tissues besides the young leaf, trace amounts (up to 0.4%) of (*R*)-2-hydroxy-3-butenyl glucosinolate were detected, whereas 4-hydroxyindol-3-ylmethyl glucosinolate was detected at trace levels in the flower and in the younger leaves. There was no indication of significant compositional differences among the plants used in this study.

The total amount of glucosinolates in different organs varies enormously (Fig. 2), being highest in the buds and young, expanding leaves (>150 μmol g dry $weight^{-1}$), at intermediate levels (100–150 μmol g dry $weight^{-1}$) in flowers, the upper stems, and middle leaves, and lowest (10–50 μmol g dry $weight^{-1}$) in the cotyledons, first true leaves, and lowest part of the stem. All of these tissues, including stems, flowers, and leaves of all ages

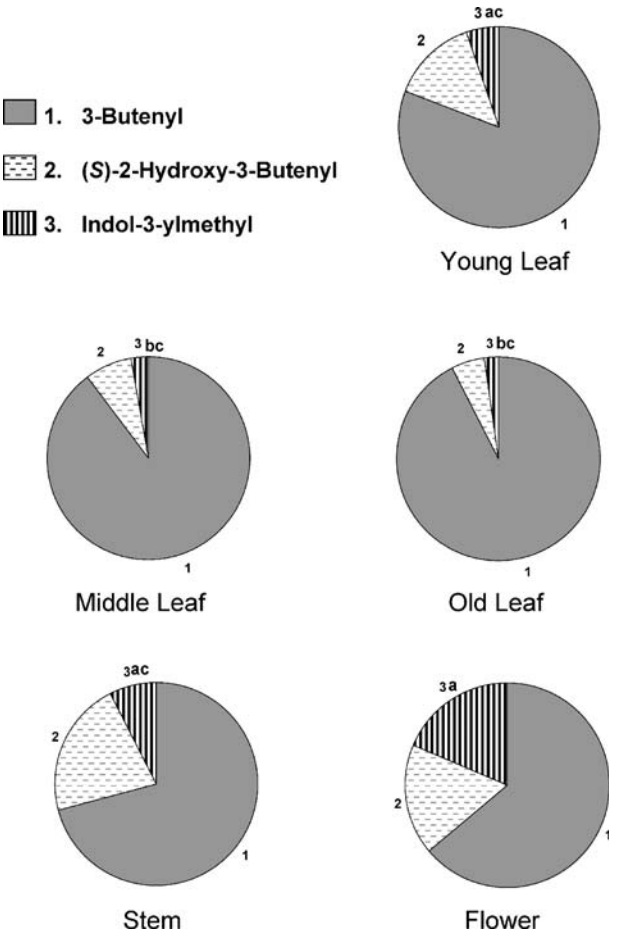


Fig. 1 Glucosinolate composition of above-ground organs of *S. purpurea*, all of which are eaten by the desert locust. Values represent the means of nine samples; for each organ type, three samples were harvested from each of three different plants. Indol-3-ylmethyl glucosinolate varied significantly ($P<0.05$) among organs with differences indicated by *letters* in the figure as determined by ANOVA followed by a Student–Newman–Keuls test on the means of the raw data before conversion to percentages for the pie chart. There were no significant differences in the means of 3-butenyl glucosinolate or (*S*)-2-hydroxy-3-butenyl glucosinolate among the organs analyzed

were eaten by the desert locust under the conditions of our experiments. Insects that had no previous experience with *S. purpurea* were observed to first eat older leaves then proceed to younger leaves when the older leaves were consumed.

Glucosinolate Hydrolysis Products of S. purpurea The biological activity of glucosinolates depends on their myrosinase-catalyzed degradation to various products. To analyze what desert locusts might naturally encounter during feeding on *S. purpurea*, leaves were mechanically macerated and allowed to autolyze in water for 5 min so that the endogenous glucosinolate pool was exposed to the native myrosinase as well as any other glucosinolate-modifying enzymes present in the plant. The main hydrolysis product in all plants analyzed (≥56%) was the epithionitrile, 3-epithiobutyl cyanide [3-(thiirane-2-yl)propane nitrile] (Fig. 3) demonstrating that *S. purpurea* possesses an epithiospecifier protein (Lambrix et al. 2001; Burow et al. 2006). The amount of 3-epithiobutyl cyanide was higher in the young leaf as compared to the old leaf, flowers, and stems ($P<0.05$ by one-way ANOVA followed by Student–Newman–Keuls test). 3-Butenyl isothiocyanate, considered more toxic than the corresponding nitrile and epithionitriles, was also formed, but this comprised less than 7% of the total glucosinolate hydrolysis products under all conditions. Low amounts of

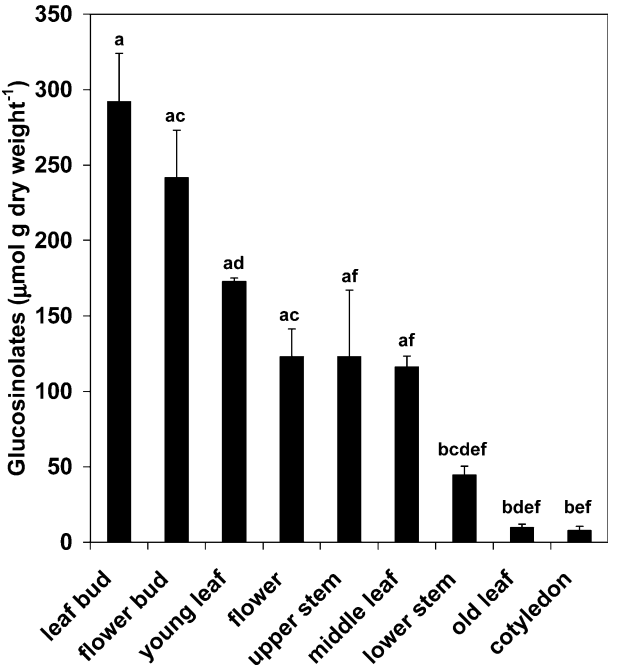


Fig. 2 Total glucosinolate content of *S. purpurea* above-ground tissues of a 5-week-old flowering plant. Values are the means±SE for three samples per tissue. Significant differences ($P<0.05$) among organs are indicated by different *letters above bars* and were determined by Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's test

indoleacetonitrile (1.6–4.2%), (*S*)-2-hydroxy-3-epithiobutyl cyanide (0.4–7.1%), and 3-butenyl cyanide (1.2–1.6%) were also produced by the plant.

Glucosinolate Metabolites Found in the Desert Locust To get an overview of the glucosinolate metabolism of the desert locust, insects were allowed to feed on *S. purpurea*, and the guts and feces were analyzed for the presence of possible glucosinolate metabolites, including glucosinolates with modified side chains, desulfoglucosinolates, and the various hydrolysis products found in the plant. The main metabolites observed (99% of total in both the gut and feces) were desulfoglucosinolates. The remaining metabolites were hydrolysis products, primarily 3-epithiobutyl cyanide. These data suggested that desert locusts possess a glucosinolate sulfatase activity in the gut similar to that demonstrated in *Plutella xylostella* (Ratzka et al. 2002).

Demonstration and Characterization of GSS in Desert Locust Guts Cell-free extracts of the alimentary canal of locusts fed with *S. purpurea* were able to convert intact glucosinolates into desulfoglucosinolates. Activity was linear for 5 min incubation time at a concentration of 0.5–20 µg protein ml^{-1}. The assay was primarily studied with allyl glucosinolate (sinigrin) as substrate, but the enzyme was also shown to be active to a varying degree with all of the glucosinolates found in *S. purpurea*: 3-butenyl-, (*S*)-2-hydroxy-3-butenyl-, and indol-3-ylmethyl-glucosinolate, as well as benzyl- and *p*-hydroxybenzyl glucosinolate (Table 1). The preparation yielded a K_m for sinigrin of 11 mM and a V_{max} of 109 µmol min^{-1} mg $protein^{-1}$, and a K_m for (*S*)-2-hydroxy-3-butenyl glucosinolate of 113 mM, and a V_{max} of 764 µmol min^{-1} mg $protein^{-1}$. The enzyme displayed a sharp pH optimum at 6.5 (range tested: 2.5–11; half-maxima: 5.8 and 7.7), which corresponds closely to the pH of the gut (6.2 in locusts fed solely *S. purpurea*). It was inhibited strongly by Na_2SO_3 and slightly by Na_2HPO_4 and NaF (Fig. 7). GSS activity was detected in the head, foregut,

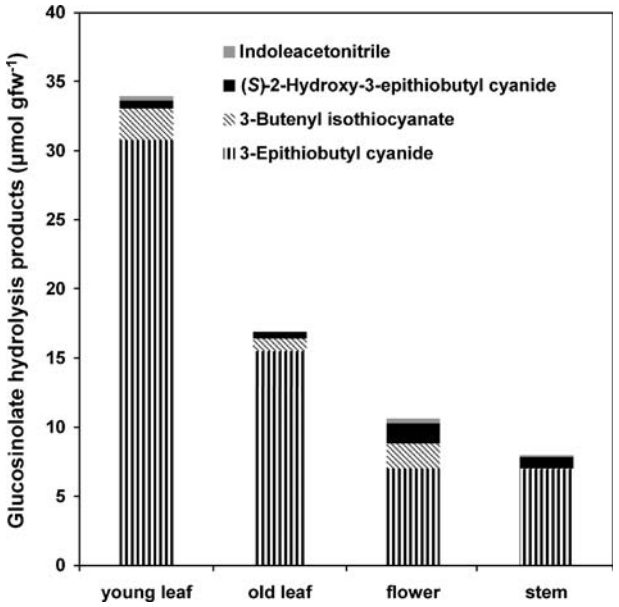


Fig. 3 Glucosinolate hydrolysis products of *S. purpurea*. Values represent the means of three samples each from three different plants. Hydrolysis of 3-butenyl glucosinolate results in the formation of two stereoisomers of 3-epithiobutyl cyanide and hydrolysis of (*S*)-2-hydroxy-3-butenyl glucosinolate results in the formation of two stereoisomers of (*S*)-2-hydroxy-3-epithiobutyl cyanide. Our chromatographic analysis did not differentiate between these stereoisomers. 3-Butenyl cyanide was detected at trace levels in this experiment but is not depicted in this figure because of its much lower level relative to 3-butenyl isothiocyanate. One-way ANOVA with a post hoc Student–Newman–Keuls test indicated a significant difference ($P<0.05$) between the amount of the major product 3-epithiobutyl cyanide in the young leaf as compared to the other organs examined

gastric caecae, midgut, and hindgut of all desert locusts at similar levels (data not shown). It was also present in all larval stages when fed with *S. purpurea* as well as in both male and female adults (Fig. 4). The enzyme was extremely stable, retaining 75% of its activity after 3 months of storage at room temperature as compared to boiled controls and losing no activity after 3 months of storage at 4 or −20°C. The extract could be heated to 70°C for 10 min with no loss of activity. The stability of this enzyme may be due its location in the highly proteolytic environment of the insect gut.

Table 1 Glucosinolate sulfatase activity in extracts of desert locust guts with various glucosinolate substrates

Glucosinolate	GSS Activity (µmol min^{-1} mg $protein^{-1}$)
Allyl	9.92±1.1
3-Butenyl	8.52±0.9
Indol-3-ylmethyl	7.92±0.9
p-Hydroxybenzyl	7.59±0.5
(*S*)-2-Hydroxy-3-butenyl	6.04±0.9
Benzyl	3.8±0.2

Each value is the mean of at least three replicate assays. All assays were performed with 1 mM of respective glucosinolate substrate.

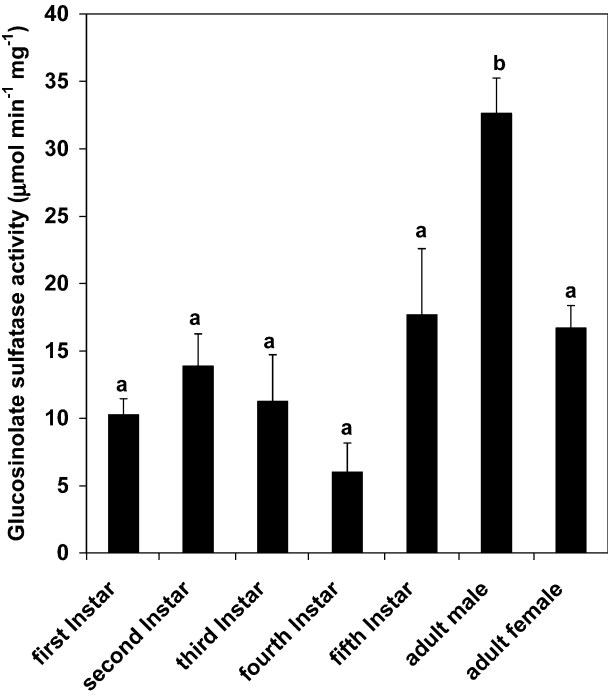


Fig. 4 Glucosinolate sulfatase activity during larval development of desert locust. Values represent the mean±SE for three assays each comprising a whole body extract of three locusts for the first through fifth instars and a gut extract of three locusts for the adult male and female. Significant differences ($P<0.05$) between developmental stages are indicated by different *letters above bars* and were determined by one-way ANOVA followed by a Student–Newman–Keuls test

Induction of GSS in Gut Upon Introduction of Glucosinolates to a Nonglucosinolate Containing Diet The desert locust is a generalist feeder that feeds on *S. purpurea* when this plant is abundant in its local environment but feeds on other nonglucosinolate-containing plants as well (Ghaout et al. 1991; Culmsee 2002). To determine whether its glucosinolate sulfatase activity was constitutive or induced upon ingestion of glucosinolates, adult females were fed on a diet of wheat for 3 days before being switched to *S. purpurea* for 9 days. After 9 days, the insects were returned to a wheat diet. Insects were sampled periodically and assayed for gut GSS activity, feces desulfoglucosinolates, and feces glucosinolate hydrolysis products. The data are shown in Fig. 5. At the beginning of the experiment, when insects were fed only wheat, GSS activity was low but measurable at 2 µmol min^{-1} mg $protein^{-1}$. Upon *S. purpurea* feeding, this activity increased tenfold to 20 µmol min^{-1} mg total $protein^{-1}$ after 9 days (the difference in GSS activity in locusts feeding on wheat vs feeding on *S. purpurea* was significant at $P<0.05$). A fourfold increase was already evident after 3 days of *S. purpurea* feeding. When insects were taken off the *S. purpurea* diet and returned to wheat, the GSS activity showed a declining trend to approximately 10 µmol min^{-1} mg total $protein^{-1}$ (not statistically significant) and remained at that level for 6 days. Coincident with the rise in GSS activity during feeding on *S. purpurea*, desulfoglucosinolates were detected at high levels in the feces of locusts. Only trace levels of desulfoglucosinolates were observed in the feces of insects before *S. purpurea* feeding. These were probably carried over from feeding on Chinese cabbage (*Brassica pekinensis*) before the start of the experiment. Similarly, when the insects were switched from *S. purpurea* to wheat, desulfoglucosinolates continued to appear in the feces after the removal of glucosinolates from the diet.

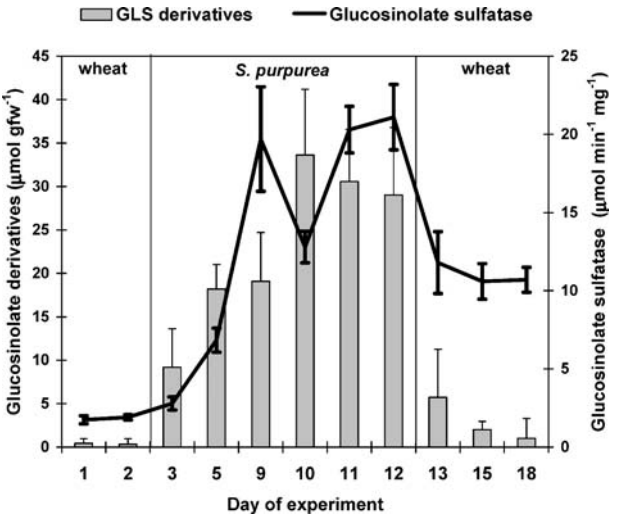


Fig. 5 Induction of GSS activity in desert locust gut and appearance of glucosinolate derivatives in feces during feeding on different diets. Values represent three measurements for each timepoint, and the experiment was repeated three times with similar results. Data for one experiment are shown. Glucosinolate derivatives consist primarily of desulfoglucosinolates, with trace amounts of 3-epithiobutyl cyanide, (*S*)-2-hydroxy-3-epithiobutyl cyanide, 3-butenyl isothiocyanate, and indoleacetonitrile. Kruskal–Wallis one-way ANOVA on Ranks followed by the Dunn's test ($P<0.05$) showed that (1) GSS activity was significantly higher in locusts fed with *S. purpurea* and locusts fed with wheat after exposure to *S. purpurea* than in insects with no prior exposure to *S. purpurea* and (2) that glucosinolate derivatives were significantly higher in feces of locusts fed with *S. purpurea* vs wheat

Relationship of GSS to Arylsulfatase Because an arylsulfatase activity has been described in the crop liquor of the desert locust (Robinson et al. 1953), it was of interest to determine its relationship to GSS. Arylsulfatases catalyze desulfonation of sulfate esters and sulfamates and have been isolated from many prokaryotes and eukaryotes including insects (Hanson et al. 2004). By using 4-nitrocatechol as a substrate, we measured a K_m in desert locust gut extracts of 1.8 mM, significantly lower than that observed for sinigrin (11 mM) or (*S*)-2-hydroxy-3-butenyl glucosinolate (113 mM). However, the V_{max} of 4-nitrocatechol is relatively low at 0.03 µmol min^{-1} mg^{-1}. When the catalytic efficiencies (V_{max}/K_m) of these activities are calculated, the GSS activity is much more efficient (19.6 for sinigrin and 6.8 for (*S*)-2-hydroxy-3-butenyl glucosinolate) than the arylsulfatase activity (V_{max}/K_m= 0.017). Furthermore, when arylsulfatase activity was assayed from insects switched from wheat to *S. purpurea*, and back to wheat again, it was observed that this activity was induced concomitantly with GSS (Fig. 6). Arylsulfatase activity showed a similar response as GSS to most of the tested inhibitors with the exception of Na_2HPO_4 (Fig. 7).

Discussion

The desert locust, *S. gregaria*, is a generalist that occasionally feeds on *S. purpurea*, a plant containing high levels of glucosinolates. As glucosinolates are deterrent and toxic to many insects (Halkier and Gershenzon 2006), it was of interest to learn how desert locusts are

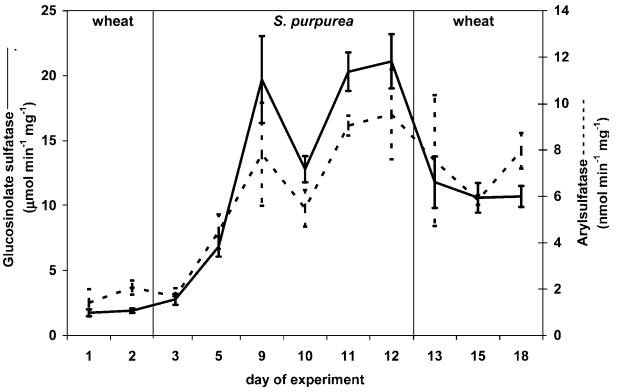


Fig. 6 Comparison of glucosinolate sulfatase and arylsulfatase activities during desert locust feeding on *S. purpurea* and wheat. Values represent mean±SE for three replicates of each assay per timepoint. Assays for each activity were optimized separately to insure linearity over the range of incubation times and protein concentrations employed

able to tolerate the presence of glucosinolates in their diet. We showed that desert locusts possess a glucosinolate sulfatase in their alimentary canal, which cleaves the sulfate group efficiently from all glucosinolates tested, rendering them inert to myrosinase activity. The resulting desulfoglucosinolates are excreted in high quantities in the feces. Glucosinolate sulfatase was first described for larvae of the diamondback moth (*Plutella xylostella*; Ratzka et al. 2002). Both the *P. xylostella* enzyme and the desert locust enzyme have broad specificity for aliphatic, indolic, and aromatic glucosinolates. The snail *Helix pomatia*, a generalist herbivore, also possesses a glucosinolate sulfatase in its gut, but it is unclear whether this enzyme is able to detoxify glucosinolates when the animal feeds on cruciferous plants (Ratzka et al. 2002).

Feeding on glucosinolate-containing tissue causes other biochemical changes in the locust gut including suppression of β-glucosidases and induction of a myrosinase activity, possibly of insect origin (Mainguet et al. 2000). However, the broad substrate specificity of the glucosinolate sulfatase, its high activity in vitro and its inducibility upon switching to a glucosinolate-containing diet accompanied by the mass appearance of desulfoglucosinolates in the feces, suggests that desulfation of glucosinolates is the major route of glucosinolate detoxification in locusts.

Although *S. purpurea* contains nearly ten times the level of glucosinolates of other crucifers, such as *Arabidopsis thaliana*, this potential toxicity is tempered by the fact that the plant generates primarily nitriles rather than isothiocyanates when tissue is macerated.

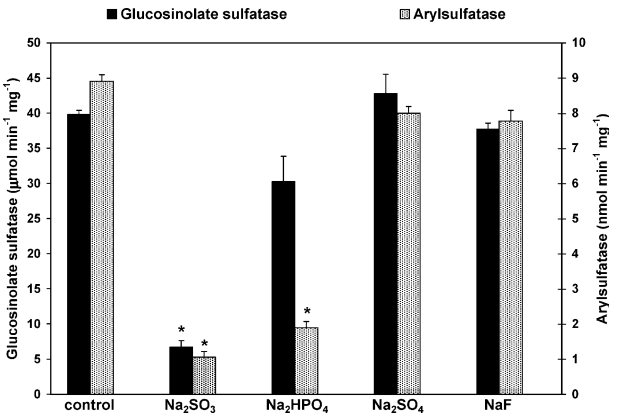


Fig. 7 Inhibitors of glucosinolate sulfatase and arylsulfatase activities in desert locust gut. Values represent the mean±SE for three replicates. Treatments significantly different ($P<0.05$) from their respective controls as determined by one-way ANOVA with a post hoc Student–Newman–Keuls test are indicated with an *asterisk*

Isothiocyanates are thought to be generally more toxic to insects than nitriles (Wittstock et al. 2003) and have been shown to be deterrent to locust feeding (El Sayed et al. 1996). However, our experiments showed that desert locusts do not encounter isothiocyanates in significant levels when feeding on *S. purpurea*.

The locust gut extract catalyzed the desulfation of 4-nitrocatechol, indicative of arylsulfatase activity, as well as the desulfation of glucosinolates. Although GSS was not purified to homogeneity in this study, the fact that the arylsulfatase activity was induced along with GSS during feeding on *S. purpurea* and that they had similar inhibitor profiles suggests strongly that the activities are catalyzed by the same protein. Arylsulfatases were first isolated based on their ability to catalyze the desulfation of model compounds and were later found to desulfate biologically more relevant molecules (Hanson et al. 2004). Arylsulfatase has been implicated in the molting of southern armyworm larvae, but its function and biological substrates in insects are not well understood (Yang et al. 1973; Pellicia et al. 1987). It is known that insects possess sulfated ecdysone conjugates (Koolman 1973) proposed to be inactive storage forms of ecdysone, and it is conceivable that arylsulfatases are involved in their metabolism. However, ecdysteroid localization is limited to the ovaries, hemolymph, and eggs, rather than the gut, and thus, it is unlikely that GSS is involved in ecdysteroid turnover. Furthermore, the induction of GSS activity by dietary factors seems to preclude its involvement in molting, as molting is a tightly regulated process, and *S. purpurea* feeding did not disrupt larval development (data not shown).

Other enzymes have been implicated in glucosinolate detoxification in insects in addition to glucosinolate sulfatase and the NSP from *P. rapae* that diverts glucosinolate hydrolysis from isothiocyanates to nitriles (Wittstock et al. 2004). Francis et al. (2005) reported that glutathione transferase activity is higher in the aphid *Myzus persicae* when it feeds on the glucosinolate-containing plants *Brassica napus* and *Sinapis alba* as compared to when it feeds on beans. Unfortunately, this study does not provide direct chemical evidence of glucosinolate detoxification because the enzymes were assayed with artificial substrates, such as 1-chloro-2,4-dinitrobenzene, rather than with glucosinolates or their products (Francis et al. 2005). Wadleigh and Yu (1988) have provided in vitro evidence for a glutathione transferase that conjugates isothiocyanates with glutathione in midgut extracts of the larvae of the cabbage looper (*Trichoplusia ni*) and the fall armyworm (*Spodoptera frugida*) but did not demonstrate the presence of these conjugates in larval feces. In animals, it is known that isothiocyanates are conjugated to glutathione (Zhang et al. 1995) before excretion as a mercapturic acid conjugate. The presence of an insect myrosinase has been reported in the cabbage aphid *Brevicoryne brassicae* (Francis et al. 2002; Husebye et al. 2005); however, as yet, no evidence has been presented that this myrosinase participates in glucosinolate detoxification in vivo. It seems rather to function in the insect's own defense against predators. On the contrary, our work and the study of Ratzka et al. (2002) on *Plutella xylostella* glucosinolate sulfatases, as well as the results of Wittstock et al. (2004) on NSP, provide strong evidence for direct glucosinolate detoxification by insects.

When first offered *S. purpurea*, desert locusts were observed to regulate their glucosinolate exposure by feeding preferentially on older tissues with lower levels of glucosinolates. This behavior may be necessary to prevent toxicity until glucosinolate sulfatase activity is sufficiently induced. A tenfold induction in GSS was observed after switching from wheat to *S. purpurea* in this study. Induction of detoxification enzymes in the insect gut is a common response to the presence of toxic plant secondary metabolites in the diet. For example, the bruchid *Callosobruchus maculatus* tolerates toxic thiosulfinates and disulfides produced by leek (*Allium porrum*) by inducing the activity of a glutathione-

S-transferase implicated in detoxification of these defensive sulfur compounds (Dugravot et al. 2004). Specific cytochrome P450s are induced in the midgut of tobacco hornworm larvae (*Manduca sexta*) that detoxify nicotine and aldrin (Snyder and Glendinning 1996) and in parsnip webworm (*Depressaria pastinacella*) and swallowtail caterpillars (*Papilio* spp.) that detoxify furanocoumarins (Li et al. 2004b; McDonnell et al. 2004). Transcription of members of the furanocoumarin-metabolizing cytochrome P450 *CYP6B* family is induced in all species of *Papilio* examined to date during feeding on furanocoumarin-containing plants, and the magnitude of the induction correlates positively with the amount of furanocoumarins ingested, which varies depending on the feeding preferences of individual species (Cohen et al. 1992; Li et al. 2001). The induction of a detoxification enzyme when the insect feeds on a plant containing its substrate, rather than its continuous presence in the gut, may function to minimize the metabolic costs of processing food (Schoonhoven et al. 2005). Thus, it is not surprising that the glucosinolate detoxifying enzyme of the desert locust, glucosinolate sulfatase, is seen only in substantial amounts after initial feeding on glucosinolate-containing plants.

The inducibility of detoxification enzymes may be a common trait in generalist feeders, like the desert locust. In comparing generalist and specialist feeding herbivores, generalists are expected to encounter a larger variety of plant defense metabolites and thus may have evolved a broader detoxification capacity. However, this does not preclude generalists and specialists from employing similar mechanisms of detoxification. Indeed, furanocoumarins are detoxified by the same subfamily of cytochrome P450s (*CYP6B*) in both generalist and specialist lepidopteran species (Li et al. 2007). Generalist lepidopterans appear to possess a greater variety of cytochrome P450s with broader substrate specificity and a greater degree of inducibility (Li et al. 2004a, 2007). Nevertheless, the basic mode of detoxification is similar. This is also true for glucosinolates where both the specialist diamondback moth (Ratzka et al. 2002) and the generalist desert locust metabolize glucosinolates via desulfation.

Acknowledgments We thank Karsten Seidelmann and Sigi Hertel for providing us with *S. gregaria*, Mohamed Lemine for the gift of *S. purpurea* seeds, Tamara Krügel and Andreas Weber for growing the plants, and Michael Reichelt for substrate preparation and maintenance of the HPLCs and GCs, as well as for helpful discussions. The Max Planck Society provided financial support.

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Keywords Allelochemical · *Brassica oleracea* · Glucosinolates · *Pieris rapae* · *Pteromalus puparum* · Parasitism

Introduction

Plants have evolved defenses against herbivores that use a wide array of direct mechanisms (Karban and Baldwin 1997). Direct defense mechanisms include morphological adaptations that impede herbivore colonization and movement on the plant, such as hairs, waxy leaf surfaces, or sticky secretions (van Dam and Hare 1998; Muller and Riederer 2005). Most plants also produce secondary metabolites that affect herbivore behavior and development. Allelochemicals may function as feeding deterrents for generalist herbivores (Akhtar and Isman 2004) or exhibit toxic effects on the development and physiology of herbivores by reducing their rates of growth and increasing mortality (Giamoustaris and Mithen 1995; Wold and Marquis 1997; van Dam et al. 2000). On the other hand, many herbivores have evolved specific metabolic adaptations that enable them to detoxify or excrete allelochemicals and their breakdown products (Ratzka et al. 2002; Wittstock et al. 2004). Still other species utilize plant allelochemicals as feeding and/or oviposition stimulants (Carter and Feeny 1999; Renwick and Lopez 1999) or sequester them as a putative defense against predators and parasitoids (Hunter 2000; Muller et al. 2001).

Several studies have found that there is considerable genetic variation in the expression of secondary plant metabolites, even in populations that are located in close proximity to each other (Fox 1981; Moyes et al. 2000; Agrawal 2005). This variation is thought to represent an adaptive response to a suite of selection pressures that may vary even over limited spatial and/or temporal gradients (Moyes et al. 2000). Biotic selection pressures, such as attack from herbivores and pathogens, and abiotic selection pressures, such as exposure to wind or access to water and nutrients, may play an important role in shaping the evolution of direct defenses in plants (Agrawal 2005). Competition with other plants may also be an important factor resulting in a trade-off between growth and defense (van Dam et al. 2000).

Most researchers now agree that natural enemies of arthropod herbivores, such as parasitoids and predators, must also be considered in models examining the evolution of direct defense strategies in plants (Price et al. 1980). This approach has generally argued that natural enemies, by regulating herbivore populations, can act as important selective agents by reducing the need for plants to invest in chemical defenses when these are costly to maintain (van Dam et al. 2000; Ode et al. 2004). However, one of the major problems with this argument is that it assumes that the development of natural enemies is independent of plant quality, thus ignoring that natural enemies obtain their nutrition indirectly from the plant through the herbivore. A number of studies have reported that allelochemicals in the diet of hosts or prey negatively affect the development of their parasitoids and predators (Barbosa et al. 1986, Duffey et al. 1986; Gunasena et al. 1990; Traugott and Stamp 1996; Havill and Raffa 2000; reviewed by Harvey 2005 and Ode 2006). Further, the effects of allelochemicals may even be manifested in the fourth trophic level (Orr and Boethel 1986; Harvey et al. 2003; Soler et al. 2005), showing that they can work their way up through the food chain.

In this study, we compared the development of the small cabbage white butterfly, *Pieris rapae* L. (Lepidoptera: Pieridae), and its gregarious ectoparasitoid, *Pteromalus puparum* L. (Hymenoptera: Pteromalidae) when the herbivore was reared on three wild populations and one cultivar of cabbage, *Brassica oleracea* L. (Capparales: Brassicaceae). Previous work found that there were significant differences in the expression of glucosinolates in wild

cabbage plants among the different Dorset populations (Mithen et al. 1995; Moyes et al. 2000). Domestication in *B. oleracea* has also resulted in a significant reduction of foliar levels of glucosinolates (Benrey et al. 1998). We included a cabbage cultivar with low levels of glucosinolates compared to the wild Dorset populations. *Pieris rapae* is a specialist herbivore that feeds exclusively on plants producing glucosinolates (Mithen et al. 1995; Renwick and Lopez 1999). Glucosinolates and their breakdown products act as feeding deterrents or exhibit detrimental effects on the growth and development of herbivores, pathogens, and nematodes (Potter et al. 1999; Li et al. 2000; Buskov et al. 2002). On the other hand, they are also known to act as oviposition and feeding stimulants for specialist herbivores such as *Pieris rapae* (van Loon and Schoonhoven 1999; Renwick and Lopez 1999).

We compared pupal weight of *Pieris rapae* caterpillars that were reared in the laboratory on the three wild strains and cultivar of *B. oleracea*. We then examined adult wasp body mass and development time in *Pteromalus puparum* when reared from pupae of *Pieris rapae*. Finally, we measured levels of glucosinolates in foliage of *B. oleracea*. The main aim of the experiment was to determine if there are differences in plant quality among the different strains of *B. oleracea*, based on foliar levels of glucosinolates, and if these are reflected in patterns of herbivore and parasitoid development.

Methods and Materials

Plants Seeds of *B. oleracea* were collected from three populations growing on or close to chalk cliffs along the south coast of Great Britain, near Swanage, Dorset. Two of the populations (Winspit, Kimmeridge) are located less than 5 km from each another; the third (Old Harry) occurs approximately 10 km east of the Winspit population. The coordinates for each of the populations are: Old Harry (50°38′N, 1°55′E), Kimmeridge (50°35′N, 2°03′E), and Winspit (50°34′N, 2°02′E). In addition, a cultivated strain of *B. oleracea* (Brussels sprout) cv. Cyrus was included.

Seeds from the different strains were germinated in the first week of October, 2005. Seedlings were transferred to 2.1-l pots filled with potting soil consisting of approximately 30% sand, 5% clay, and 65% peat. Plants were grown at The Netherlands Institute of Ecology, Heteren, in a greenhouse at 22±2°C, 40–80% RH, with a photoperiod of at least 16 hr. If the light dropped below 500 μmol photons m^{-2} sec^{-1} during the 16 hr photoperiod, supplementary illumination was supplied by high-pressure mercury lamps. Plants were watered daily. Starting when 4-wk old, the plants were fertilized once a week with 0.5 regular Hoagland solution, which was applied to the soil. All *B. oleracea* plants were 7-wk old when they were used in experiments.

Insects Pieris rapae feeds exclusively on plants in the Order Capparales that produce glucosinolates, and especially on species of the Brassicaceae, and is considered to be a major pest in cabbage crops in many parts of the world. During its development, *Pieris rapae* passes through five instars before pupating on leaves or stems of the plant. *Pteromalus puparum* is known to attack pupae in several families of butterflies, including species of the Nympahlidae and Pieridae (Lei et al. 1997; Barron et al. 2004; Wold-Burkness et al. 2005). It is considered to be an important biological control agent of *Pieris rapae* (Lee and Heimpel 2005). The parasitoid must first host-feed before initiating oogenesis. Host feeding occurs when the female parasitoid creates a wound in the host cuticle by repeatedly inserting and removing her ovipositor over the course of several minutes. After this time, haemolymph begins oozing

through the wound and is consumed by the parasitoid. Eggs usually are produced within 48 hr of a host-feeding bout.

All insects used in the experiments originated from cabbage fields in the vicinity of Wageningen. Cultures of all herbivores were maintained in the laboratory on Brussels sprouts cv. Cyrus for many years and reared in climate rooms at 22±2°C, 40–80% r.h, and a light regime of L16:D8. *Pteromalus puparum* were obtained from pupae of *Pieris rapae* and the closely related large cabbage white butterfly *P. brassicae* in June, 2005 and were thereafter reared on pupae of *P. brassicae*. Parasitized butterfly pupae were maintained in large Petri dishes (20-cm diameter) and were monitored until adult parasitoid emergence. Emerged adult wasps were then placed in separate Petri dishes and were provided with water and honey *ad libitum*. For parasitism, we used females that were 5- to 10-d old after adult emergence.

Development of Pieris rapae and Pteromalus puparum on B. oleracea To compare the effects of plant quality on herbivore and parasitoid development, *Pieris rapae* and *Pteromalus puparum* were reared on three wild strains and one cultivar of *B. oleracea*. To obtain eggs of *Pieris rapae*, one plant from each strain was placed in an insect-rearing cage containing at least 50 adult butterflies for 4 hr. After this period, plants were removed from the cages and placed in a rearing room until the eggs hatched. When the larvae reached the second instar (=L2), they were transferred to new plants with a distribution of nine larvae per plant. A total of six plants were used to rear 54 *Pieris rapae* per strain. During their development, caterpillars were allowed to develop and move around freely on the plants until they reached the final instar. When larvae had developed into late fifth instar, they were transferred to plastic containers (15×10×10 cm) with some leaf material from the plants they were previously feeding on. After pupation, the pupae were carefully collected and weighed on a Cahn C-29 microbalance (Cahn Instruments, Cerritos, CA, USA; accuracy, ±1 μg). A subset of these weighed pupae was used for parasitism by the parasitoid, *Pteromalus puparum* (see below). In addition to pupal mass, egg-to-pupa development time and survival (egg-to-pupa) were recorded when reared on the different plant strains. Plants with insects were maintained in a greenhouse compartment under the same conditions as described in "Plant" section.

A subset of 15 pupae reared from each strain of *B. oleracea* was selected to be parasitized by *Pteromalus puparum*, ensuring that there were no significant differences in mean pupal fresh mass (Table 2). This avoided the potential problem of disentangling the effects of pupal mass and plant strain on parasitoid development. Individual, mated parasitoid females that had previously been host-fed were provided with single pupae of *Pieris rapae* originating from one of the four *B. oleracea* strains in large Petri dishes for 24 hr (thus, the number of female parasitoids used was the same as for the number of pupae in Table 2). After this time, the female wasps were removed, and the parasitized butterfly pupae were monitored several times daily until adult parasitoid emergence. Newly emerged wasps were sexed, killed by freezing, and then placed in a preheated oven at 70°C for 3 d to obtain dry-mass data. All wasps within broods were then weighed on the Cahn balance. Development times were recorded as the number of days between the termination of the initial 24-hr parasitism period and adult eclosion. Unfortunately, some parasitoids failed to parasitize *Pieris rapae* pupae (i.e., adult butterflies enclosed), and these were eliminated from analyses. The sample size (hosts parasitized) was therefore: Cyrus=12, Kimmeridge=7, Old Harry=14, and Winspit=7.

Glucosinolate Analyses As an indicator of direct plant defense, we measured glucosinolate concentrations in leaf tissues of *B. oleracea* from the different strains. All plants that were used for insect development, six plants per plant strain, were also sampled for glucosinolate analysis. When leaf samples were taken, the damaged plant groups had been exposed to

Pieris rapae feeding for 7 d. Two discs, 7-mm diameter, were punched per leaf, one from each central part of a leaf half, and six fully unfolded leaves were sampled per plant. Leaf discs were pooled per plant in 2-ml Eppendorf vials and frozen in liquid nitrogen immediately after sampling and stored at −80°C. Samples were later freeze-dried and pulverized. Fifty-milligram aliquots of freeze-dried material were weighed in 2-ml centrifuge tubes. Glucosinolates were extracted and purified as described by van Dam et al. (2004). Glucosinolates were separated on a reverse phase C-18 column (Alltima C-18, 3 μm, 150×4.6 mm, Alltech,Deerfield, IL, USA) on high performance liquid chromatography (HPLC; Dionex, Sunnyvale, CA, USA) with an acetonitrile-water-gradient. Detection was performed with a Dionex PDA-100 photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, MO, USA) was used as an external standard. Peaks were integrated at 229 nm for which standard response factors have been defined. Leaf tissues of *B. oleracea* contain aliphatic and indole glucosinolates. The aliphatic glucosinolates have a side chain length derived from a propyl (C3) or butyl (C4) group. These different glucosinolates (indoles, C3, C4) were identified based on their retention times and UV spectra compared to those of pure compounds (sinigrin, Sigma, St, Louis, MO., USA), glucotropaeolin, glucobrassicin (kindly provided by M. Reichelt, MPI for Chemical Ecology, Jena, Germany), or compared to a certified oil seed reference (EC Community Bureau of Reference BCR-367R, Fluka, Buchs, Switzerland). For a more detailed description of the method, see van Dam et al. (2004).

Statistical Analyses Survival of *Pieris rapae* was analyzed by using a chi-square test. Data on development time and pupal mass of *Pieris rapae*, brood size of *Pteromalus puparum*, and glucosinolate content in the different *B. oleracea* strains were analyzed by analysis of variance (ANOVA) with plant strain as factor. Normality and homogeneity of variance were checked by inspection of the residuals after model fitting and transformed if necessary. Brood size was square-root transformed; glucosinolate contents were log-transformed. The Tukey–Kramer method was used for multiple comparisons of means. For *Pteromalus puparum* development time and adult weight, multiple datapoints were associated to each host pupa. Therefore, these data were analyzed with a mixed model (REML) in Genstat 7. The analysis calculates Wald-type *F*-statistics and can process different sources of variation, enabling the use of all individuals rather than using averages per host (Piepho et al. 2003). Data were analyzed with strain, sex (male or female), and the interaction as fixed factors and host pupa as random factor. Predicted means and standard errors and individual comparisons estimated after model fitting were used for presentation of the results.

Results

Glucosinolate Profiles Total glucosinolate levels were higher in the wild populations than in the cultivar (Fig. 1). The rank order of total glucosinolates levels in the different populations is: Cultivar < Kimmeridge < Old Harry < Winspit. Glucosinolate concentrations of individual compounds differed significantly among the populations, except for glucoraphanin, progoitrin, and 4-hydroxy glucobrassicin (Table 1). In Winspit and Old Harry plants, the aliphatic glucosinolate with the highest concentration was gluconapin, although levels of gluconapin were significantly higher in Winspit than in Old Harry plants. In the cultivar, glucoiberin dominated the aliphatic glucosinolate profile. In contrast, Kimmeridge plants contained low levels of aliphatic glucosinolates (<2 μg/g DW all aliphatic glucosinolates combined). The wild populations contained significantly higher levels of the indole

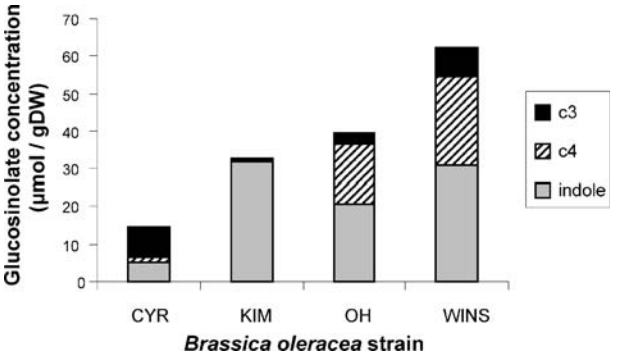


Fig. 1 Mean total glucosinolate concentrations (N=6) in foliage of one cultivar (*CYR*) and three wild populations of *B. oleracea* (*KIM*, *OH* and *WINS*) after 7 d of feeding by *Pieris rapae*. Total glucosinolates are divided into propyl (*C3*) derived aliphatic, butyl (*C4*) derived aliphatic, and indole glucosinolates

glucosinolate glucobrassicin than the cultivar. Furthermore, the Kimmeridge and Winspit plants contained higher amounts of neoglucobrassicin than the other two populations.

Development of Unparasitized Pieris rapae on Different Strains of Brassica oleracea Survival of *Pieris rapae* varied with plant strain in *B. oleracea* ($\chi^2_3 = 9.69$, P=0.021). Herbivore survival to pupation was almost 90% or higher in all strains with the exception of Kimmeridge plants, where only 76% of caterpillars successfully pupated (Fig. 2a).

Pupal mass varied with plant strain in *B. oleracea* plants ($F_{3,186}$=44.58, P<0.001; Fig. 2b). Pupae were largest when larvae of *Pieris rapae* were reared on the cultivar (Cyrus) and Old Harry plants and smallest when reared on Kimmeridge plants (Fig. 1). Plants from the Winspit population were of intermediate quality. Larval development time in *Pieris rapae* also varied with plant strain ($F_{3,186}$=4.99, P=0.002). *Pieris rapae* larvae took much longer to reach pupation on Kimmeridge plants than on Cyrus or Old Harry plants (Fig. 2c). Development time was also slightly prolonged on Winspit plants.

Table 1 Glucosinolate concentrations (mean±SE µmol/g DW) in foliage of four different populations of *Brassica oleracea* damaged by *Pieris rapae* for 7 d

Populations	Kimmeridge (N=6)	Old Harry (N=6)	Winspit (N=6)	Cultivar (N=6)	ANOVA $F_{3,24}$	P Value
C3 aliphatic glucosinolates						
Glucoiberin	0.22±0.14a	1.32±0.83a	0.44±0.44a	6.25±0.55b	17.23	<0.001
Sinigrin	0.38±0.19a	1.63±0.77ab	7.12±2.10b	1.92±0.41ab	7.33	0.002
C4 aliphatic glucosinolates						
Glucoraphanin	0.11±0.07a	4.33±2.66a	1.11±0.71a	0.84±0.09a	2.18	0.12
Gluconapin	0.00±0.00a	8.63±3.27b	20.01±2.10c	0.00±0.00a	30.74	<0.001
Progoitrin	0.30±0.24a	3.19±2.73a	2.53±2.06a	0.49±0.06a	0.65	0.59
Indole glucosinolates						
Glucobrassicin	19.22±2.98a	18.26±3.43a	19.35±6.18ab	4.80±0.65b	4.77	0.01
Neoglucobrassicin	12.11±5.26b	1.95±1.46a	11.36±3.60b	0.00±0.00a	8.71	0.007
4-hydroxy GBC	0.14±0.04a	0.20±0.04a	0.12±0.03a	0.19±0.01a	1.51	0.24
4-methoxy GBC	0.09±0.01a	0.07±0.03a	0.12±0.04a	0.23±0.01b	6.63	0.003

ANOVA was used on ln(x+1) transformed data to reveal differences between the populations. Different letters within a row denote significantly different means (Tukey–Kramer multiple comparisons, P<0.05).

N Number of samples per population; *GBC* glucobrassicin

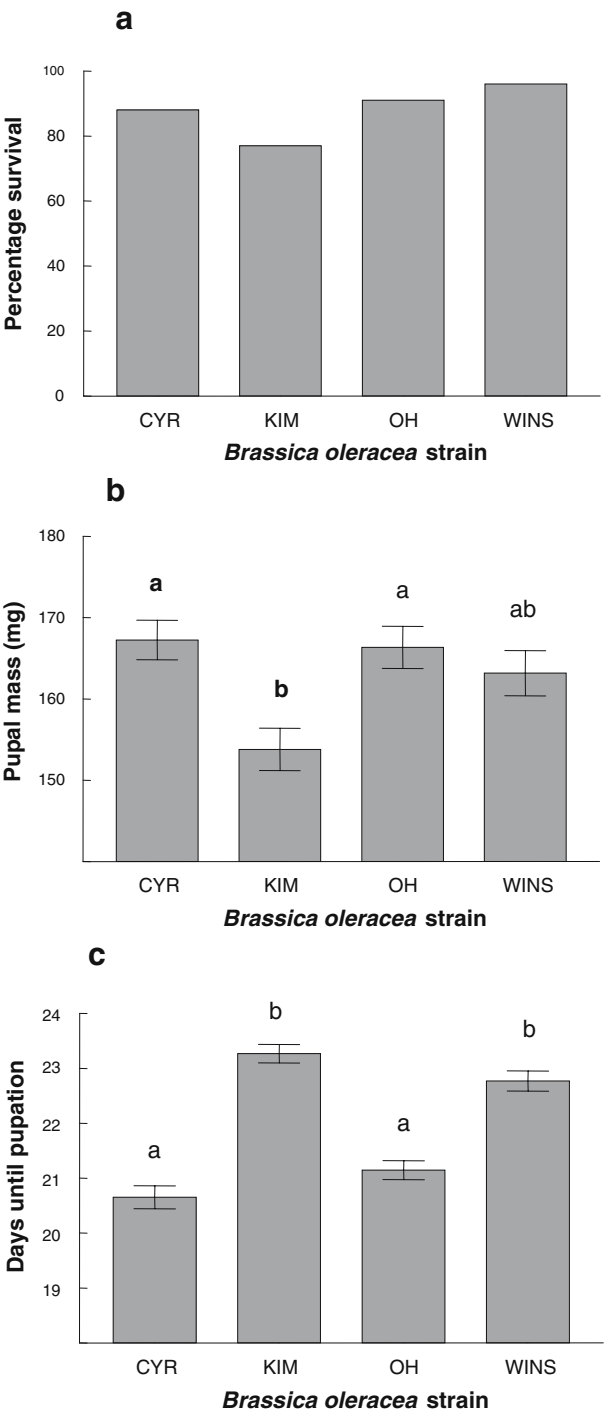


Fig. 2 Development of *Pieris rapae* reared on three wild populations and a cultivar of *Brassica oleracea*. **a** Percentage survival from egg to pupa; **b** Mean (±SE) pupal mass (mg), and **c** mean (±SE) development time from oviposition until pupation (d). *Bars with the same letters* are not significantly different (Tukey's tests, $P>0.05$). Sample sizes: Cyrus, $N=48$, Kimmeridge $N=41$, Old Harry $N=49$, Winspit $N=52$

Table 2 Mean (±SE) wet pupal masses (mg) of *Pieris rapae* parasitized by *Pteromalus puparum*

Plant Population	Sample Size	Mean Pupal Wet Mass (mg ± SE)
Cyrus	12	165.97±3.13 a
Kimmeridge	7	161.67±8.00 a
Old Harry	14	162.83±4.65 a
Winspit	7	162.99±7.52 a

Wet weights with the same letter are not significantly different ($P>0.05$).

Pupal Mass of Pieris rapae Parasitized by Pteromlaus puparum Pupal mass of *Pieris rapae* selected for parasitism by *Pteromalus puparum* did not vary significantly across the different *B. oleracea* strains ($F_{3,36}=0.12$, $P=0.95$; Table 2). Irrespective of plant strain, the mean pupal mass of *Pieris rapae* that produced parasitoids was consistently between 160 and 165 mg (Table 1).

Primary Brood Size and Development of Pteromalus puparum on Pieris rapae pupae A total of 904 parasitoids emerged from 40 host pupae that had been reared on the four populations of *B. oleracea*. Brood size in *Pteromalus puparum* did not vary significantly when emerging from *Pieris rapae* pupae reared in the different strains of *B. oleracea* ($F_{1,36}=0.56$, $P=0.64$; Fig. 3). Typical brood sizes were in the range of 20–26 wasps per host pupa (Fig. 3).

Body mass in *Pteromalus puparum* varied among strains of *B. oleracea* (Wald=11.48, $P=0.009$), but the interaction between strain and sex was also highly significant (Wald=26.09, $P<0.001$). Overall, female wasps were larger than male wasps resulting in a significant difference between offspring sex (Wald=289.24, $P<0.001$). Parasitoid body mass varied among strains in male wasps (Wald=14.02, $P=0.003$; Fig. 4a) but not in female wasps (Wald=4.76, $P=0.19$; Fig. 4b). In male parasitoids, the largest wasps developed in Cyrus and Old Harry plants, whereas the smallest developed in plants from the Kimmeridge population. Winspit plants were of intermediate quality in this respect. Although there was no effect of plant strain in female wasps, the trend was similar to that observed in male wasps (Fig. 4a,b).

Egg-to-adult development time in *Pteromalus puparum* did not vary significantly among the different strains of *B. oleracea* (strain, Wald=2.89, $P=0.41$; strain × sex, Wald=0.58; $P=0.90$; Fig. 4c,d) but did vary with offspring sex (Wald=14.89, $P<0.001$). *Pteromalus puparum* is protandrous, with female wasps taking longer to complete their development than male wasps. Development in *Pteromalus puparum* takes about 3 wk to be completed.

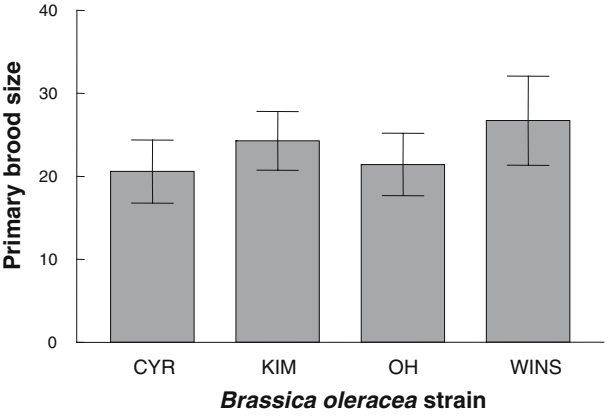


Fig. 3 Mean (±SE) brood size of *Pteromlaus puparum* emerging from pupae of *Pieris rapae* that were in turn reared on three wild populations and a cultivar of *Brassica oleracea*. Sample sizes (number of *Pieris rapae* pupae producing adult *Pteromlaus puparum* wasps from each plant population): Cyrus $N=12$, Kimmeridge $N=7$, Old Harry $N=14$, Winspit $N=7$

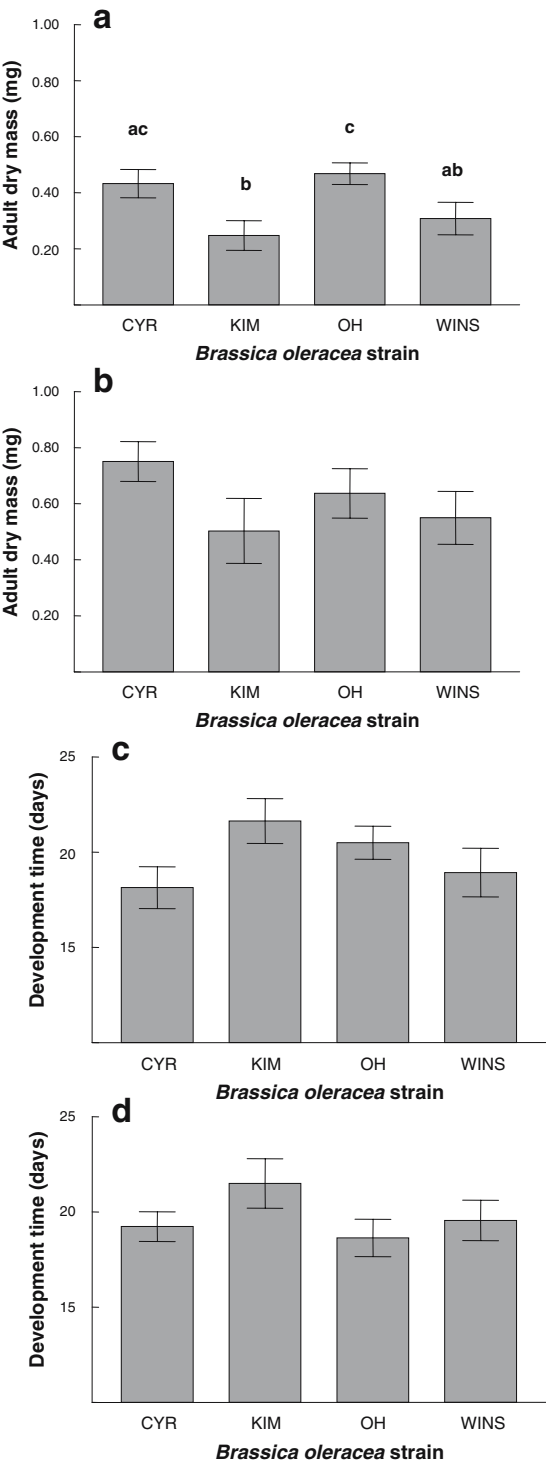


Fig. 4 Development of *Pteromlasus puparum* emerging from pupae of *Pieris rapae* that were in turn reared on three wild populations and a cultivar of *Brassica oleracea*. Mean (±SE) adult mass (mg), of **a** males wasps and **b** female wasps; mean (±SE) development time from oviposition until pupation (d) of **c** male wasps, and **d** female wasps. Means and standard errors are estimated after model fitting. *Bars with the same letters* are not significantly different (Tukey's tests, $P>0.05$). Sample size as in Fig. 2

Discussion

We found that pupal mass of *Pieris rapae* and adult body mass of *Pteromalus puparum* varied nonlinearly with total glucosinolate levels in the four strains of *B. oleracea*. However, both the herbivore and its parasitoid appeared to be similarly affected by differences in plant quality. In both species, the largest individuals developed on the cultivar and Old Harry plants, whereas body mass was greatly reduced when developing on plants from the Kimmeridge population. Winspit plants were of intermediate quality. In *Pieris rapae* (but not *Pteromalus puparum*), development time also was extended for larvae reared on Kimmeridge plants. The similarity in the effect of plant strain on the body mass of *Pieris rapae* and *Pteromalus puparum* reveals that qualitative constraints on the herbivore were passed from the second to the third trophic level. Several other studies have reported variable effects of plant quality, probably mediated by allelochemicals, on the performance of insect parasitoids and predators (Campbell and Duffey 1979; Barbosa et al. 1986, Gunasena et al. 1990; Havill and Raffa 2000; Harvey et al. 2003, 2005, 2007).

If herbivore and parasitoid performance were positively correlated with total glucosinolate levels, we would have predicted that the rank order of plant quality would be cultivar > Kimmeridge > Old Harry > Winspit. However, this was not the case, suggesting that total glucosinolate content does not determine plant quality for *Pieris rapae*. Glucosinolates and especially their hydrolysis products, (iso)thiocyanates, are known to be active against insect herbivory (Li et al. 2000; Wittstock et al. 2004). However, *Pieris rapae* has developed an effective detoxification mechanism, which redirects the hydrolysis of glucosinolates into less toxic nitriles through the activity of a nitrile specifier protein (Wittstock et al. 2004). This glucosinolate detoxification system may render *Pieris rapae* relatively insensitive to high levels of glucosinolates.

The only glucosinolate compound that affected the performance of *Pieris rapae* was the indole glucosinolate, neoglucobrassicin. High concentrations of neoglucobrassicin in plants from the Kimmeridge and Winspit populations corresponded to reduced biomass and prolonged development of the herbivore when reared on these two populations. Indole glucosinolates are known to be induced by herbivory and fungal infection and to affect the growth and development of insect herbivores deleteriously (Rostás et al. 2002; Irwin et al. 2003). Levels of primary metabolites, such as nitrogen and amino acids, were not measured in this study. Although levels of these are unlikely to be limiting in cruciferous plants for herbivores (Soler et al. 2005), significantly different levels of nitrogen have been recorded in wild and cultivated species of crucifers (Slansky and Feeny 1977). In addition, levels of the enzyme myrosinase, which catalyzes the hydrolysis of glucosinolates, may also have varied in the different populations (Mithen 2001; Fahey et al. 2002).

In wild populations of *B. oleracea* growing along the south coast of England, previous work in the field also found that there was considerable variation in the expression of glucosinolates (Mithen et al. 1995; Moyes et al. 2000). However, although glucosinolates measured in plants from the same populations as those used in this study exhibited similar differences among populations (e.g., with Winspit plants containing the highest and Kimmeridge plants the lowest concentrations), overall total levels of glucosinolates in all three were higher than recorded here (Moyes et al. 2000).

The effect of plant quality on parasitoid size in *Pteromalus puparum* was more pronounced in male than in female wasps. For instance, adult male wasps emerging from *Pieris rapae* that had fed on Kimmeridge plants were almost 50% smaller, in terms of dry mass, than conspecifics emerging from hosts that had fed on the cultivar or Old Harry plants. By contrast, this difference in females, which was not significant statistically, was

less than 25%. This variation may be attributable to differing levels of selection in male and female wasps to achieve optimal body size. Although large size presumably benefits both sexes, large females are assumed to gain more in terms of fitness than males because egg production is more constrained in small females than sperm production is in small males (Charnov 1982).

As mentioned earlier, Wittstock et al. (2004) reported that *Pieris rapae* larvae do not sequester glucosinolates during feeding, but instead utilize a nitrile specifier protein in the gut to break them down into more harmless nitriles that are excreted with the feces. This suggests that internal host fluids and tissues should be less toxic for natural enemies that attack insects such as *Pieris rapae* than is the case with hosts or prey that actively sequester plant toxins into their own body tissues (Hunter 2000). However, other studies with herbivores that detoxify or excrete harmful plant allelochemicals have shown that the development of parasitoids may still be correlated negatively with levels of allelochemicals in host diet (Campbell and Duffey 1979; Barbosa et al. 1986, Harvey et al. 2007). In some parasitoids, trace amounts of secondary plant metabolites have also been found in their by-products, including cocoon silk and/or the meconium (Barbosa et al. 1986; Bowers 2003).

In addition to the wild *B. oleracea* populations, genetic variation in the expression of allelochemicals has been studied extensively in only a few species of wild plants, including both forbs and trees (Fritz et al. 2003; Lawrence et al. 2003; Ode et al. 2004; Zangerl and Berenbaum 2005; Loney et al. 2006). Interpopulation differences in the expression of allelochemicals may be driven by the intensity of selection from herbivores, particularly if natural enemies are scarce or absent (Ode et al. 2004; Zangerl and Berenbaum 2005). Reciprocally, this can have profound effects on plant selection and performance in generalist herbivores, although studies have focused mainly on mammals, such as brush-possums in Australia (O'Reilly-Wapstra et al. 2005; Loney et al. 2006). However, population-related differences in secondary plant chemistry have also been shown to affect the performance of arthropod natural enemies such as parasitoids and predators (Harvey et al. 2005; this study). One of the major shortcomings of existing studies is that detailed information is lacking on the possible factors underlying variation in phenotypic traits such as defense in plants. This should be the focus of future research.

A recent study by Gols et al. (2007) revealed that even under controlled conditions, levels of glucosinolates in shoots of two species of cultivated crucifers changed dramatically over the course of a single growing season. As a result, the development of two insect herbivores and their parasitoids also differed in each of three replicates that were performed (Gols et al. 2007). This suggests that, had further replicates been performed, the effects described here may also have varied temporally in line with changes in plant quality. Nonetheless, under a given set of conditions, we have shown that population-related differences in plant quality can have significant and uniform effects on the development of a herbivore and its parasitoid. Further studies are being planned in which spatial and temporal aspects of interactions involving natural and manipulated populations of *B. oleracea* and its lepidopteran herbivore–parasitoid complexes are investigated. These studies will help to explain factors accounting for the dramatic differences in secondary chemistry among populations of wild cabbage along the south England coast and will shed further light on the relative importance of biotic and abiotic processes shaping direct defense plant strategies in plants.

Acknowledgments The authors thank Leo Koopman and Andre Gidding at Wageningen University, NL, for supplying larvae of *Pieris rapae*, James Bullock of the Centre for Ecology and Hydrology in Dorset, England, for supplying seeds from wild cabbage populations, Nicole van Dam for interpreting glucosinolate profiles and Wim van der Putten and Roxina Soler for their constructive comments on an earlier version of the manuscript.

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protecting against future infection by pathogens (Friedrich et al. 1996). The plant elicitor benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), also known as acibenzolar-*S*-methyl and Actigard®, is a functional analog of salicylic acid that activates SAR in both monocots and dicots (Gorlach et al. 1996; Lawton et al. 1996). Foliar application of BTH induces pathogen response genes typically associated with SAR and provides protection against an array of bacterial, fungal, and viral plant pathogens in cotton and other plants (Friedrich et al. 1996; Gorlach et al. 1996; Inbar et al. 1998; Colson-Hanks et al. 2000; Maxson-Stein et al. 2002; Bokshi et al. 2003; Zhu et al. 2003; Baysal et al. 2005). Further, foliar application of BTH to tomato foliage was shown to decrease leafminer and whitefly densities (Inbar et al. 1998; Nombela et al. 2005) and aphid population growth (Boughton et al. 2006). Because of the efficacy of BTH in protecting against phytopathogens and some types of insects in several cropping systems, this elicitor, along with other SAR activators, may be used with increasing frequency in integrated pest management (IPM) systems (reviewed in the study of Vallad and Goodman 2004).

While SAR-activated plants may be protected from phytopathogens, they may also be more vulnerable to chewing herbivores. Lepidopteran larvae feeding on naïve plants induce a variety of directly toxic and/or antinutritive compounds through the jasmonate-dependent wound response pathway (reviewed in the work of Duffey and Stout 1996; Bi et al. 1997a; Chen et al. 2005; reviewed in the paper of Felton 2005). The compounds induced in response to herbivore feeding reduce lepidopteran growth and can alter lepidopteran susceptibility to insect pathogens, depending on the system (Ali et al. 1998; Hoover et al. 1998b). SAR induction can inhibit this jasmonate-dependent response pathway (reviewed in the study of Felton and Korth 2000; Thaler et al. 2002; Cipollini et al. 2004) and may make plants previously challenged by phytopathogens more suitable hosts for lepidopteran larvae than naïve plants or plants previously damaged by chewing herbivores (Stout et al. 1999; Inbar et al. 2001; reviewed in Rostas et al. 2003). Although the apparent potential for SAR induction to influence lepidopteran susceptibility to insect pathogens and other natural enemies is great, the extent to which there is compatibility of SAR with natural enemies of lepidopterans has not been fully explored (but see the work of Rostas and Hilker 2003 for a review).

One class of insect natural enemies are the baculoviruses, a group of orally infectious, dsDNA viruses of arthropods that commonly infect lepidopteran larvae (reviewed in the paper of Bonning 2005). The family Baculoviridae is divided into the genera Granulovirus and Nucleopolyhedrovirus (NPV) (Theilmann et al. 2005). NPVs are found in nature as environmentally resistant proteinaceous occlusion bodies (OB) on host plant foliage, and susceptible larvae become infected when they consume at least one OB (reviewed in the study of Bonning 2005). Upon ingestion, the OB is degraded in the alkaline midgut of the host (Pritchett et al. 1982), releasing occlusion-derived virions, which pass through the peritrophic matrix and infect midgut epithelial cells (Granados and Lawler 1981). NPVs replicate in the nucleus of infected cells; progeny virions bud from infected midgut cells and enter associated tracheolar cells and hemocytes, spreading the infection to other tissues, eventually killing the host.

Most baculoviruses are highly host specific, infecting, at the extremes, from 1 to 40 lepidopteran species (reviewed in the paper of Cory and Myers 2003). Because of their high degree of host specificity, baculoviruses have been considered candidate pest control agents (reviewed in the work of Szewczyk et al. 2006). The potential of baculoviruses as bioinsecticides has not been fully realized due in part to the high cost of production, inactivation by ultraviolet light, and a slow speed of kill. As these issues are addressed, baculoviruses could be implemented more frequently as a part of IPM strategies. Thus, it is important to assess how baculoviral efficacy is influenced by other components of IPM. In

this study, we analyzed whether cotton foliar peroxidase activity and quantities of phenolic compounds change after SAR induction by BTH. Furthermore, we evaluated the influence of SAR induction on pathogenicity of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) to *Heliothis virescens* larvae.

Methods and Materials

Insect Rearing Heliothis virescens eggs were acquired from the North Carolina State University Insectary, from a colony that started in 1997 (Raleigh, NC, USA). Larvae were grown in groups through the third stadium on semisynthetic diet (Southland Products, Lake Village, AR, USA) in wax-lined paper cups (Xpedx, Harrisburg, PA, USA) at 25°C and a 16L:8D photoperiod.

Plant Growing Conditions Cotton, *Gossypium hirsutum* cv. Maxxa, seeds were donated by the California Planting Cotton Seed Distributors (Bakersfield, CA, USA). Seeds were sown individually in sterile soil in plastic pots, fertilized at planting with slow-release 14–14–14 fertilizer, and grown in an Conviron® environmental chamber under a day:night temperature regime of 29:24°C and a 16L:8D photoperiod.

Plant Treatments BTH (Actigard®) was provided by Syngenta/Novartis (Greensboro, NC, USA). A 0.056-g/l solution of BTH (Actigard® 50WG) in ultrapure water was sprayed to run off on upper leaf surfaces of six-node cotton plants. Control plants were left untreated or sprayed with ultrapure water. Plants to be treated were removed from the chamber for application of solutions to prevent drift to control plants. The concentration of 0.056 g/l used is similar to concentrations of 0.035 g/l (Colson-Hanks and Deverall 2000) and 0.05 g/l (Jabaji-Hare and Neate 2005) applied to cotton foliage in previous studies, yet is much lower than the 0.8 g/l applied by Inbar et al. (2001). We found higher concentrations than those used here phytotoxic to this cultivar of cotton.

Four days after treatment, the top two fully expanded leaves of all control and BTH-treated plants were removed. Foliage removed for use included leaves 5 and 6 (the cotyledons were not counted) from eight-node plants. A subsample of intact leaves from the water and BTH treatment groups was frozen in liquid nitrogen and stored at −80°C for phytochemical assays. Foliage from untreated and BTH-treated plants was fed to larvae to determine the influence of SAR activation on pupal weight and viral mortality.

Foliar Assays Peroxidase activity was measured in untreated and BTH-treated cotton foliage using a guaiacol/hydrogen peroxide substrate by measuring the change in absorbance at OD_{470}, as described previously (Bi et al. 1997a), except that the assay was modified for use with a microplate reader. Peroxidase activity is reported as change in absorbance, with one unit of activity=0.001 ΔOD_{470} per milligram of foliage per minute. Three untreated and three BTH-treated plants were used for the peroxidase assay, with three measurements conducted per plant. Leaves from positions 5 and 6 were combined and used from each plant.

Phenolic compounds were extracted from water-treated and BTH-treated cotton foliage in 50:50 acetone to water at 60°C for 20–24 hr. This extract was used for the following assays. Total phenolics were measured with the Folin-Ciocalteau reagent with caffeic acid as the standard at OD_{720} (Singleton and Rossi 1965). Dihydroxyphenolics were measured by using the 0.5% diphenylborinic acid–ethanolamine complex with chlorogenic acid as the

standard at OD_{365} (Broadway et al. 1986). Condensed tannins were measured with the vanillin–HCl assay with catechin as the standard at OD_{500} (Price et al. 1978; Bi et al. 1997a). A SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA) was used for all of the above spectrophotometric measurements. For the dihydroxy- and total phenolic assays, five water-treated and three BTH-treated plants were used, with three measurements made per plant. For the condensed tannin assay, three water-treated and two BTH-treated plants were used, with three measurements made per plant. Leaves from positions 5 and 6 were combined and used from each plant. The same foliar samples were used for all of the above phenolic assays.

Influence of SAR Induction on Time to Pupation and Pupal Weight Newly molted second instars of *H. virescens* were transferred individually to 30 ml plastic cups containing artificial diet or foliage from BTH-treated or untreated cotton plants and were allowed to feed *ad libitum*. Larvae were maintained at 28°C until pupation, and fresh foliage was provided as needed. Weight was recorded at pupation, with 31–38 pupae weighed per treatment group. Foliage was taken from 50 BTH-treated and 50 control plants to feed larvae; within each treatment group, foliage was randomly distributed among larvae. Foliage was harvested from treated plants 4–7 d after BTH application from nonsquaring plants. We have found that foliar peroxidase activity remains significantly elevated in BTH-treated plants relative to controls for at least 7 d after treatment. Foliage was concurrently harvested from control plants.

Virus Preparation AcMNPV-hsp70/*lacZ*, provided by Suzanne Thiem (Michigan State University, East Lansing, MI, USA), was amplified in *H. virescens* larvae. Occlusion bodies (OB) were harvested from virus-killed cadavers and partially purified by using ultrapure water as described previously (Hoover et al. 1995), quantified with a hemacytometer, and stored at 4°C in neutrally buoyant 60% glycerol with 0.002% sodium azide (Engelhard et al. 1994). We used a genetically modified construct of AcMNPV because, although this experiment did not require lacZ expression, concurrent experiments did, and this gene does not affect viral performance.

Influence of SAR Induction on Viral Mortality of H. virescens Newly molted fourth instars of *H. virescens* were transferred individually to a 30-ml plastic cup containing artificial diet or foliage from BTH-treated or untreated cotton plants and were allowed to feed for 8 hr. Eight hours postmolt was used because it was the earliest time point at which the midgut was full of plant material or artificial diet for all larvae based on the time at which frass was first produced. The susceptibility of *H. virescens* larvae to fatal viral infection decreases as larvae age within an instar, so controlling for age postmolt is essential (Washburn et al. 1995). Foliage was taken from 35 BTH-treated and 35 control plants to feed larvae. Within each treatment group, harvested foliage was randomly distributed among larvae.

After feeding for 8 hr, larvae were microinoculated *per os* with 95 OBs of AcMNPV-hsp70/*lacZ* in a 1-ml aliquot by using a PAX 100 microapplicator (Burkard Scientific, Middlesex, UK) holding a 1-ml tuberculin syringe with 32-gauge blunt needle (Popper Precision Instruments, Lincoln, RI, USA). After inoculation, the larvae were returned to their respective preinoculation food of artificial diet or foliage from BTH-treated or untreated cotton plants. Insects were maintained at 28°C and were checked daily for mortality or pupation. All cotton-fed larvae were transferred to artificial diet after molting to the fifth instar for ease of handling. This transfer does not influence mortality since larvae clear midgut infections during the first molt after viral challenge (Washburn et al. 1995). Two trials were performed with larvae fed with BTH-treated and untreated cotton foliage,

and three trials were performed with larvae fed with artificial diet. The first trial included all three treatment groups; subsequent trials included artificial diet and either BTH-treated or untreated cotton foliage. Some 25 to 30 artificial diet-fed larvae and 40–50 cotton-fed larvae were inoculated for each trial.

Data Analysis Data were analyzed with SAS (SAS Institute 2003). Measurements of foliar peroxidase activity and quantities of phenolic compounds were compared between control and BTH-treated plants using the general linear model with Tukey post hoc analysis (proc GLM). The effects of food and BTH treatment on *H. virescens* time to pupation and pupal weights were also determined using the general linear model with Tukey post hoc analysis (proc GLM). The influence of food, BTH application, and their interaction on the proportion of *H. virescens* larvae killed by virus was assessed using the analysis of variance for categorical data (proc CATMOD) (Zar 1999).

Results

Foliar BTH treatment increased cotton peroxidase activity 2.5-fold over that observed in untreated plants (F=6.49, df=1, P=0.029; Fig. 1), indicative of induction of systemic acquired resistance (SAR) (Inbar et al. 2001). Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester application also resulted in large increases in certain phenolic classes. For instance, we observed a nearly twofold increase in total phenolics (F=31.62, df=1, P=0.011; Fig. 2) and a 2.4-fold increase in condensed tannins in foliage of BTH-treated plants (F=12.58, df=1, P=0.038; Fig. 3) compared with water-treated controls. However, application of BTH did not significantly affect the foliar concentration of dihydroxyphenolics (F=1.31, df=1, P=0.335; Fig. 3).

Consumption of control foliage by *H. virescens* larvae increased time to pupation, relative to artificial diet and BTH-treated foliage (F=24.82, df=2, P<0.001) (Fig. 4). Interestingly, the developmental rate of larvae fed with artificial diet and those fed with BTH-treated foliage was similar (Fig. 4). In contrast, consumption of induced foliage did not impact pupal weight (Fig. 5) nor did we observe a marked increased in consumption of BTH-treated cotton foliage relative to untreated foliage (data not shown). The pupal weights of larvae fed with untreated or induced foliage were similar (F=0.66, df=1, P=0.421).

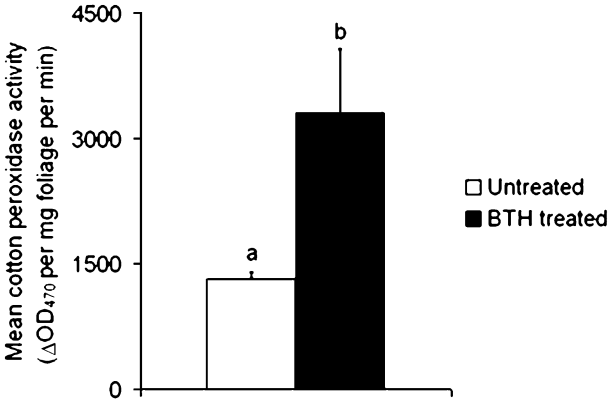


Fig. 1 Mean peroxidase activity measured at 96 hr post-spray, shown as change in activity per milligram of cotton foliage per minute. *Error bars* represent one standard error of the mean; N=3 plants per treatment. The general linear model with Tukey post hoc analysis was used to compare peroxidase activities; *small letters* indicate significant differences between treatments (F=6.49, df=1, P=0.029)

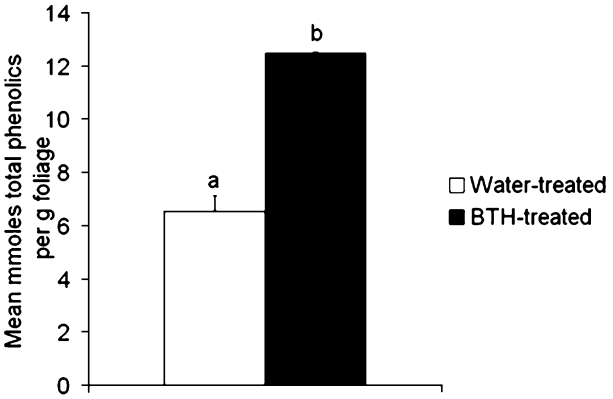


Fig. 2 Mean concentrations of total phenolics measured at 90 and 96 hr post-spray with water or BTH. *Error bars* represent one standard error of the mean. N=5 plants for water treatment and 3 for BTH treatment. The general linear model with Tukey post hoc analysis was used to compare levels of phenolics; *small letters* indicate significant differences between treatments (F=31.62, df=1, P=0.011)

Further, larvae fed with artificial diet displayed dramatically higher pupal weights than larvae fed with either treated or untreated cotton foliage (F=95.24, df=2, P<0.001; Fig. 5).

Although some classes of phenolics and peroxidase activities were induced by BTH treatment, consumption of induced foliage did not significantly influence *H. virescens* larval susceptibility to a well-known natural enemy of this host, the baculovirus AcMNPV (Fig. 6). Larvae fed with BTH-treated cotton foliage experienced a similar level of viral mortality as larvae fed with untreated foliage (χ^2=1.11, df=1, P=0.292). However, when larvae consumed induced or uninduced foliage, they were 1.3-fold more resistant to mortal infection than larvae fed with artificial diet (χ^2=21.64, df=1, P<0.001; Fig. 6).

Discussion

Application of BTH to foliage of a number of plant species is known to induce SAR, conferring resistance to phytopathogens (Friedrich et al. 1996; Inbar et al. 1998; Maxson-

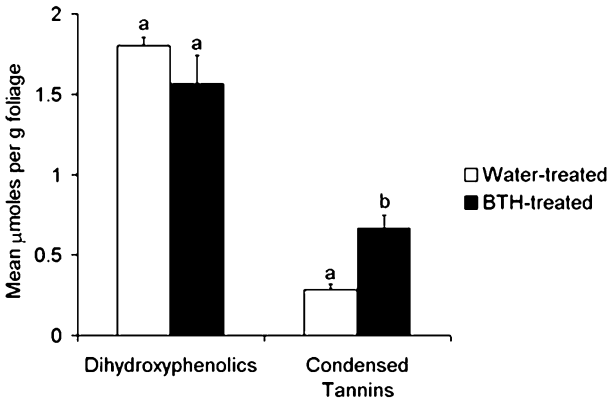


Fig. 3 Mean concentrations of dihydroxyphenolics and condensed tannins measured at 90 and 96 hr post-spray with water or BTH. *Error bars* represent one standard error of the mean. For the dihydroxyphenolics assay, N=5 plants for water treatment and 3 for BTH treatment. For the condensed tannin assay, N=3 plants for water treatment and 2 for BTH treatment. The general linear model with Tukey post hoc analysis was used to compare levels of phenolics; *small letters* indicate significant differences between treatments (dihydroxyphenolics: F=1.31, df=1, P=0.335; condensed tannins: F=12.58, df=1, P=0.038)

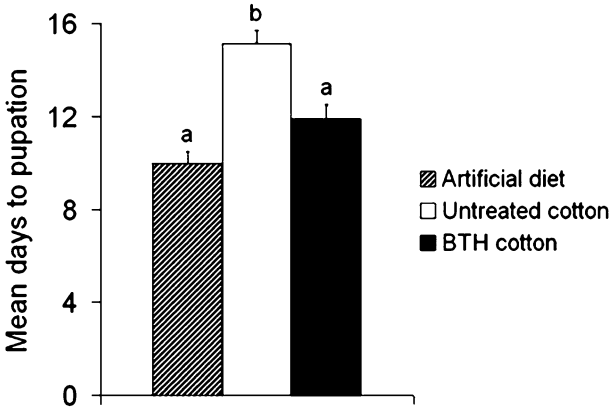


Fig. 4 Mean time to pupation of *H. virescens* fed with artificial diet or foliage from untreated or BTH-treated cotton plants from second instar to pupation. *Error bars* represent one standard error of the mean; N=35 larvae fed with artificial diet, 37 fed with untreated foliage, and 22 fed with BTH-treated foliage. The general linear model with Tukey post hoc analysis was used to compare times to pupation; *small letters* indicate significant differences between treatments (F=24.82, df=2, P<0.001)

Stein et al. 2002; Bokshi et al. 2003; Baysal et al. 2005) and, in some cases, to phloem-feeding insects such as aphids and whiteflies (Nombela et al. 2005; Boughton et al. 2006). However, SAR activation by BTH or salicylic acid in tomato or cotton foliage did not influence growth rate or survival of larval *Helicoverpa zea* (Bi et al. 1997b; Stout et al. 1999; Inbar et al. 2001). We found that although SAR activation by application of BTH to cotton plants increased foliar peroxidase activity, condensed tannins, and total phenolics, consumption of induced foliage did not affect *H. virescens* pupal weight compared with water-treated or untreated foliage. Likewise, Inbar et al. (2001) reported that while BTH applied to cotton foliage activated SAR—as evidenced by increased foliar peroxidase, chitinase, and β-1,3-glucanase—consumption of foliage from BTH-treated plants did not alter growth or survival of larval *Helicoverpa armigera*. Similarly, *H. zea* larvae fed with foliage from cotton plants treated with either salicylic acid or methyl salicylate grew as well as those that fed on untreated foliage (Bi et al. 1997b).

Increases in foliar phenolic levels after activation of the SAR pathway by BTH have been recently reported in strawberries (Hukkanen et al. 2007). Here, we found a similar

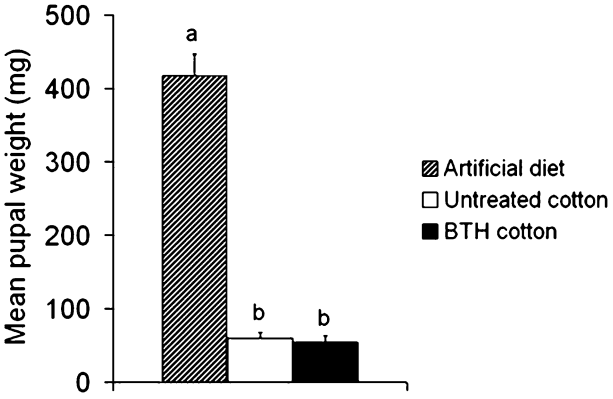


Fig. 5 Mean pupal weight of *H. virescens* fed with artificial diet or foliage from untreated or BTH-treated cotton plants from second instar to pupation. *Error bars* represent one standard error of the mean; N=35 larvae fed with artificial diet, 37 fed with untreated foliage, and 22 fed with BTH-treated foliage. The general linear model with Tukey post hoc analysis was used to compare pupal weights; *small letters* indicate significant differences between treatments (artificial diet versus cotton foliage: F=95.24, df=2, P<0.001; untreated versus BTH-treated cotton: F=0.66, df=1, P=0.421)

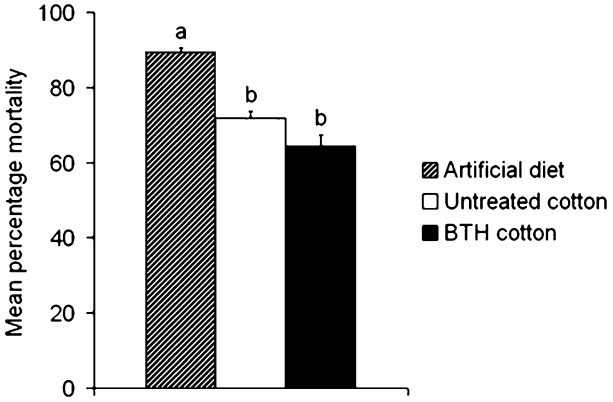


Fig. 6 Mean percentage mortality of *H. virescens* larvae fed with artificial diet or foliage from untreated or BTH-treated cotton plants before AcMNPV inoculation. The influence of food and BTH treatment on larval mortality was determined using the analysis of variance for categorical data (artificial diet versus cotton foliage: χ^2=21.64, *df*=1, *P*<0.001; untreated versus BTH-treated cotton: χ^2=1.11, *df*=1, *P*=0.292). *Error bars* represent one standard error of the mean; *N*=23–33 larvae fed with artificial diet, 38–53 larvae fed with untreated foliage, and 45–49 larvae fed with BTH-treated foliage. Two trials were performed with larvae fed with BTH-treated and untreated cotton foliage, and three trials were performed with larvae fed with artificial diet

result in BTH-treated cotton foliage. However, despite the induction of condensed tannins in treated foliage, we did not observe any influence of BTH treatment on *H. virescens* pupal weight. There is a large body of literature on cotton and other plants, suggesting that condensed tannins reduce growth in lepidopteran larvae (Feeny 1968; Chan et al. 1978; Kopper et al. 2002; Nomura and Itioka 2002; but see the study of Ayers et al. 1997 for a review). Also, while consumption of condensed tannins extracted from cotton has been shown to reduce larval mass in artificial diet experiments (Chan et al. 1978; Klocke and Chan 1982; Reese et al. 1982; Navon et al. 1983), the opposite is true *in planta*, where greater levels of foliar condensed tannins have either been positively correlated with or shown not to influence larval weight (Hanny 1980; Hedin et al. 1983; Mulrooney et al. 1985; McColl and Noble 1992; Smith et al. 1992). Although our results agree with previous experiments that used plant foliage, the tannin levels observed in the plants used in our experiments were fairly low, possibly resulting from suboptimal lighting (Dudt and Shure 1994). Thus, we cannot rule out the possibility that tannin levels in our plants were simply too low to have an effect on *H. virescens* pupal weight. At the same time, inconsistencies in experimental outcomes between artificial diet and *in planta* are common and likely reflect the numerous differences in chemical composition between artificial diet and plant foliage. The activity of specific phenolic compounds is known to depend in large part on chemical mixture and the environment in which reactions occur (Bernays et al. 1989; Appel 1993; Duffey and Stout 1996; Hoover et al. 1998b, c).

Just as we did not observe an influence of SAR activation on growth of *H. virescens* larvae, we also saw no influence of induced foliage on *H. virescens* larval susceptibility to baculoviral infection despite significant increases in peroxidase activity and condensed tannins. Peroxidase, condensed tannins, and dihydroxyphenolics have been implicated in altering baculoviral pathogenicity to lepidopteran larvae (Felton et al. 1987; Keating et al. 1988; Felton and Duffey 1990; Young et al. 1995; Hoover et al. 1998a, b; Ali et al. 1999). In particular, dihydroxyphenolics and tannins decrease mortal baculovirus infection in artificial diet experiments (Keating et al. 1989; Felton and Duffey 1990; Young et al. 1995).

While these compounds were demonstrated to influence baculoviral efficacy individually, they may act differently in mixture. Hoover et al. (1998b) found that, when considered individually, both peroxidase and condensed tannins reduced mortality of *H. virescens* larvae fed with cotton foliage. However, when considered in a mixed model, peroxidase reduced larval mortality, whereas condensed tannins increased mortality (Hoover et al. 1998c). Because we measured increased levels of both of these phytochemicals in induced foliage, it is possible that the failure of induced plants to affect *H. virescens* larval mortality may be explained by offsetting influences of peroxidase and condensed tannins, as suggested by the model of Hoover et al. (1998b).

The effect of SAR may have been different if the larvae had been fed through their entire life cycle on foliage. The underlying impetus for our experimental design was to specifically address a hypothesis suggested by Hoover et al. (1998c) that states that foliar peroxidase is negatively related to larval mortality by virus during the process of initial infection. Exposure to induced foliage during the instar in which viral challenge occurs was sufficient to test the proposed hypothesis but may not reflect what would happen in the field with long-term exposure, particularly given that feeding on plants throughout an insect's life cycle can have profound effects on insect–pathogen interactions (see review by Cory and Hoover 2006).

In summary, despite significant changes in several phytochemical components in BTH-treated plants, activators of SAR are likely to be compatible with microbial control agents such as baculoviruses in an IPM system.

Acknowledgments We thank Tony Pomicter and Mike Grove for their assistance with experiments and phenolic assays and Diana Cox-Foster, Kelly Johnson, and A. Daniel Jones for their valuable discussions. We thank Syngenta for supplying the BTH. This work was a part of the dissertation of R.P. and was funded by the National Science Foundation Integrated Organismal Biology Program, Grant No. IBN-0077710.

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Keywords Behavior · Brassicaceae · Induction · Insect resistance · Mechanical wounding · Vascular connectivity · Glucosinolates · Myrosinase

Abbreviations

p-OH	benzylglucosinolate
P	hydroxybenzylglucosinolate
Fw	fresh weight of sample

Introduction

Plants defend themselves against herbivorous insects with a plethora of mechanical and chemical devices. Next to constitutive defense, plants can also respond to herbivore attack with increased metabolite production and enzyme activities (Dicke and Hilker 2003). These induced defenses are thought to have evolved as a cost-saving strategy for plants growing under nutrient limited conditions (Zangerl 2003). Specialist herbivores can show various countermeasures as adaptations to the specific constitutive metabolites of their host plants (Hartmann and Ober 2000; Agerbirk et al. 2006), and some might also be able to cope with induced defenses. However, their performance and behavior may be impacted (Agrawal and Kurashige 2003). In contrast, generalists invest in more common detoxification strategies (Wadleigh and Yu 1988; Schoonhoven et al. 1998) but lack specific adaptations. Thus, from the plants perspective, there exists a dilemma: While they defend themselves chemically against a variety of generalists, nondeterred specialists can use these metabolites as host finding and oviposition cues. Furthermore, highly adapted specialists use plant toxins for their own protection against members of the third trophic level by sequestration ("lethal plant defense paradox", Fordyce 2001). For the plant, it might be adaptive to respond differently to attack depending on the inducing organism, e.g., with regard to the degree of specialization of the feeding insect (Baldwin and Preston 1999; Schwachtje et al. 2006).

In Brassicaceae, glucosinolates and their hydrolyzing enzymes, the myrosinases, form a constitutive defense system against herbivores and microorganisms (Halkier and Gershenzon 2006). Glucosinolates consist of a thioglucoside moiety linked to a variety of amino-acid-derived side chains (Bones and Rossiter 2006). Myrosinases (thioglucoside glucohydrolases, EC 3.2.1.147) occur in various isoforms that are either soluble under nondenaturing conditions or insoluble because of complex formation with myrosinase-binding proteins (MBP; Rask et al. 2000; Eriksson et al. 2002). The myrosinase subunits of the insoluble complexes are active, and a strongly modified protocol for the quantification of insoluble as well as soluble fractions of myrosinase has been proposed (Müller and Martens 2005; Martin and Müller 2007). A variety of specialist insects developed means of circumvention and detoxification of the glucosinolate–myrosinase system (Bones and Rossiter 2006, and references therein). Larvae of the turnip sawfly *Athalia rosae* L. (Hymenoptera: Tenthredinidae), which feed on different species of Brassicaceae, sequester certain glucosinolates of their host plants within their hemolymph (Müller et al. 2001; Müller and Wittstock 2005) and can use these for their own defense (Müller et al. 2002; Müller and Brakefield 2003).

Glucosinolates and myrosinases have also been shown to be inducible in many ways (e.g., Rostás et al. 2002; Pontoppidan et al. 2005; see also references in Martin and Müller 2007). Feeding by *A. rosae* larvae induced short-term increases in levels of glucosinolates and myrosinase activities in damaged leaves of *Sinapis alba* L. cv. Salvo (Martin and Müller 2007). However, the specificity of this effect with regard to the inducing agent and its

ecological relevance for the glucosinolate-sequestering herbivore remained unclear. In general, the specificity of a plant's induction response is dependent upon the inducing agent and the feeding mode of the herbivore (Walling 2000; Dicke and Hilker 2003). Furthermore, the vascular architecture can constrain induction patterns within a plant (Orians 2005). In Brassicaceae, studies comparing induction responses to three agents, mechanical damage, specialist, and generalist feeding, have to date focused only on gene expression (Reymond et al. 2004). In pairwise comparisons, changes in gene expression and in chemical and mechanical defenses have been found (Agrawal 2000; Reymond et al. 2004; de Vos et al. 2006; Widarto et al. 2006). However, effects on myrosinases have been studied mainly in isolation from glucosinolates (but see Siemens and Mitchell-Olds 1998; Andréasson et al. 2001). Specialist behavior and performance is affected to different degrees by induced responses of plants (Agrawal 2000; de Vos et al. 2006). None of the insects investigated with regard to behavior uses plant defenses for its own protection.

The aims of this study were to investigate the specificity of induction responses of *S. alba* that we described recently (Martin and Müller 2007) and to characterize its ecological relevance for larvae and for adult females of the glucosinolate-sequestering specialist *A. rosae*. Specificity of response was investigated at different levels. At the level of induction mode, plants were treated in three different ways with either mechanical wounding, feeding by generalist caterpillars, or by the specialist larvae of *A. rosae*. At the plant level, we used a different cultivar of *S. alba* than in a previous study. We determined glucosinolate concentrations and myrosinase activities (soluble and insoluble fractions) in local as well as adjacent older and younger leaf tissues after 24 hr of induction on *S. alba* cv. Silenda plants. To investigate potential directions for signal transport, connectivity of phloem bundles between these tissues was followed with a dye. Subsequent behavioral studies with larvae and with adult females of *A. rosae* were performed to evaluate the consequences of specific induction responses of the plant on the specialist. In this way, a triple comparison of induction effects of a generalist, a specialist, and wounding was investigated with respect to both induced plant response and induced resistance against a specialist herbivore.

Methods and Materials

Planting and Induction Experiment Seeds of *S. alba* L. cv. Silenda (Kiepenkerl, Norken, Germany) were sown in mid-January 2005 and kept in a climate chamber at 25°C, 16:8 hr L/D cycle, and 70% relative humidity (light source: Osram L 58 W/25 Universal White, 4150 lumens, Osram, Munich, Germany). After 7 d, seedlings were transferred to individual pots (9 cm diameter; 7 cm height) containing unfertilized soil (peat, pH 6). After 21 d, when five to six true leaves had developed, plants received one of the following five treatments on the second youngest leaf to investigate the specificity of the plant's response: (a) one male larva of the specialist *A. rosae* (6 d after egg hatch, third instar; larval weight 32.7±1.6 mg, mean± SE, N=8) enclosed with the leaf in a muslin bag (8×10 cm, 1 mm mesh width); (b) one caterpillar of the generalist *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae; 7 d after egg hatch, third instar; caterpillar weight 21.7±1.5 mg, mean±SE, N=8) in a bag; (c) mechanical damage made by three holes (one in the morning at 10 A.M., one in the afternoon at 4 P.M., and one the next morning at 8 A.M.) punched with a cork borer (1.5 cm diameter) and the leaf enclosed in a bag; (d) a bag only; and (e) no treatment. Effects of herbivore damage on myrosinase gene expression had been shown to be more resembled by cutting with scissors than by crushing with forceps (Pontoppidan et al. 2005). Therefore, we used a cork borer as a cutting tool to remove equivalent amounts of leaf tissue. Seven to eight replicates

per treatment were prepared. Plants of all treatments were placed in random order in the climate chamber. Sawfly larvae were taken from a laboratory culture established from a field collection in Germany the previous autumn and kept on *S. alba*. Caterpillars of *S. frugiperda* were obtained from Bayer CropScience AG (Monheim, Germany) as eggs and were reared on an artificial diet based on kidney beans (modified from King and Leppla 1984).

Insects and muslin bags were removed after 24 hr, and photos were taken of treated leaves to determine the pattern and degree of damage. Samples for chemical analyses were taken from damaged leaves as well as from the flanking upper and lower leaves. Leaves were cut longitudinally, weighed, frozen in liquid nitrogen, and stored at −80°C for later analysis. This allowed us to analyze both glucosinolate and myrosinase levels from the same leaves.

Analysis of Glucosinolates Frozen samples were freeze-dried, weighed, and pulverized in a mill (Retsch, MM301, Haan, Germany). Glucosinolates were extracted in 80% methanol after addition of 20 µl of a 5 mM solution of 2-propenylglucosinolate (Merck, Darmstadt, Germany) as internal standard. Glucosinolate analysis was performed by conversion to desulfoglucosinolates as described elsewhere (Agerbirk et al. 2001). For analysis of desulfoglucosinolates, samples were subjected to HPLC analysis on a 1100 Series chromatograph (Hewlett-Packard, Waldbronn, Germany) with a quaternary pump and a 1,040 M diode array detector. Elution was accomplished on a Supelco C-18 column (Supelcosil LC-18, 250×4.6 mm, 5 µm, Supelco, Bellefonte, PA, USA) with a gradient (solvent A, water; solvent B, methanol) of 0–5% B (10 min), 5–38% B (24 min), followed by a cleaning cycle. Desulfoglucosinolates were identified by comparison of retention times and UV spectra to those of purified standards and identities that had been further confirmed by liquid chromatography/mass spectrometry (Müller et al. 2001; Müller and Martens 2005). Peaks were quantified by peak area at 229 nm (4 nm bandwidth) relative to the area of the internal standard peak, by applying the response factors as used by Brown et al. (2003) and Martin and Müller (2007).

Analysis of Myrosinase Activity Myrosinase activity was determined by photometric quantification of released glucose from the externally added substrate *p*-OH-benzylglucosinolate (obtained from glucosinolates.com, Denmark), which is the dominant glucosinolate in *S. alba*. The myrosinase extraction protocol followed Müller and Martens (2005) and Martin and Müller (2007). Frozen leaf material was pulverized in a mill, extracted in buffer (200 mM Tris, 10 mM EDTA, pH 5.5) on ice, centrifuged, and supernatants were purified via ion-exchange columns (DEAE Sephadex A-25 welled in acetic acid buffer; Sigma-Aldrich, St. Louis, MO, USA) to eliminate internal glucosinolates. Purified extracts served as source to determine soluble myrosinase activities. The remaining pellets were suspended in buffer, and two further tests were performed. Half of each pellet suspension was diluted with buffer to determine the activity of insoluble myrosinases. The other half was centrifuged, and supernatants were discarded. The resulting pellets were suspended in half of the corresponding purified extract. Thereby, a suspension was obtained with a reconstituted ratio of soluble and insoluble myrosinases similar to the crude, unpurified extract but without interfering internal glucosinolates. Soluble protein concentrations of purified extracts were determined according to Bradford (1976) and were adjusted to protein concentrations of 200 µg ml^{-1}. Both suspensions were diluted the same way as the purified extracts.

Activities of soluble and insoluble fractions of myrosinases were determined individually and together for each leaf sample on 96-well microplates. The three enzyme preparations were each added to four wells. Two wells served as test wells into which 2 mM *p*-OH-benzylglucosinolate-phosphate buffer was added as substrate to measure kinetics of glucose

release. Two additional wells served as respective background controls that received only phosphate buffer to measure levels of internal glucose and light scattering. Glucose was detected by addition of 50 µl freshly mixed color reagent, including glucose oxidase, peroxidase, 4-aminoantipyrine, and phenol (Kunst et al. 1984; Siemens and Mitchell-Olds 1998) and quantified by reading the absorbance at 492 nm on a Multiskan EX (Thermo Labsystems, Vantaa, Finland) for 45 min at room temperature (22 to 24°C). Means of the two replicate measurements were calculated after subtraction of the means of the two background controls. A glucose dilution series was included in each assay. The rates of coloration that can be impacted by ascorbic acid (Kleinwächter and Selmar 2004) were not affected by ascorbate under our assay conditions (Travers-Martin et al. unpublished data). The resulting enzyme kinetics were analyzed for a linear range of myrosinase activity. Whereas the specific activity (Units mg^{-1} protein) is used to characterize an enzyme's activation status (Moss 1984; Stranger and Mitchell-Olds 2005), the concentration of myrosinase activity in relation to tissue fresh weight is a measure of the plant tissue's capacity to produce harmful hydrolysis products. Myrosinase activity concentrations are given in nanomoles of glucose released per minute and per milligram fresh weight of sample (fw) in this study, as we focused on the ecological effects.

Analysis of Soluble Protein and Water Contents Protein concentrations were obtained from purified extracts of the myrosinase assay. Soluble protein concentration was measured in duplicate of two dilutions according to Bradford (1976). Water contents of tissues were determined after freeze-drying of samples used for glucosinolate analysis.

Behavioral Experiments with Athalia rosae To investigate the behavioral response of specialist *A. rosae* larvae to metabolite changes in plant tissues evoked by different induction modes, *S. alba* plants were induced as described above with treatments a, b, c, and d. Twenty replicates were prepared per treatment. After 24 hr of induction, bags and insects were removed, and each plant received one male *A. rosae* larva (4 d after egg hatch, third instar; larval weight 16.3±1.3 mg, mean±SE, N=80). Larvae were placed onto the soil of the pot near the base of the plant and were allowed to move freely to and on the plants. Time until first feeding and feeding sites at the first and nine subsequent time points (90, 215, 285, 350, 405, 635 min after start of the test on the same day and 1,265, 1,340, 1,430 min after start of the test on the next day) were recorded within 24 hr. To determine oviposition behavior of *A. rosae* on differently treated *S. alba*, plants were induced as described above with 14 replicates per treatment. After 24 hr of induction and after the removal of bags and insects, plants were placed into large muslin bags (20×60×10 cm, mesh width 1 mm), and each received one naive (without oviposition experience), unmated female *A. rosae* (4 d after hatching from pupae, female weight 23.1±0.4 mg, mean±SE, N=52) for 24 hr. Numbers of eggs were counted for each leaf separately. Locations of larval feeding and egg deposition within 24 hr on differently induced plants were grouped into two categories in behavioral tests. Category I included the treated leaf and all younger ones in the upper plant region because induction effects were strongest in these tissues. Category II included all older leaves below the treated leaf, where induction effects were less pronounced. A preference index of categories (PI_c) was calculated per female as [(number of eggs on category I)−(number of eggs on category II)]/total number of eggs. The extreme values of 1 and −1 represent preferences for categories I and II, respectively.

Plant Vascular Connectivity For the determination of the vascular connections of the second youngest leaf of *S. alba*, nine plants were subjected to phloem staining with

rhodamin B modified from Orians et al. (2000). Plants were wounded on the second youngest leaf with a cork borer (three holes, no time delay, 5 mm diameter). A droplet of 25 μl of a 0.25% solution of rhodamin B in nanopure water was applied to each of the wounded areas and sealed with parafilm and silicone grease (Wacker, Munich, Germany) to minimize evaporation. After 24 hr of stain relocation in the phloem, treated leaves as well as the adjacent upper and lower leaves and stem parts were harvested. Samples were freeze-dried and pulverized in a mill. Rhodamin B was removed by three subsequent extractions with 400 μl of nanopure water for leaves and 200 μl for stems. Supernatants were separated from pellets by centrifugation twice at room temperature for 10 min at 16,060×*g*. Supernatants were analyzed for rhodamin B content by reading the absorbance at 570 nm on a Multiplate EX photometer. A standard dilution series of rhodamin B was included on each microplate.

Statistical Analyses Differences in damage patterns and in induction patterns of water contents, soluble protein, glucosinolate, and myrosinase activity concentrations between treatments in organs of identical age were analyzed by one-way analysis of variance (ANOVA) followed by HSD tests for imbalanced number of subsets. Variance homogeneity was examined by Levené tests, and data were log- or squareroot-transformed where needed. Control groups without and with bag treatment were analyzed for effects of the muslin bag on induction responses by *t*-tests. Treatment effects on the number of damage sites were analyzed by Mann–Whitney *U* tests that were tied and Bonferroni corrected.

In behavioral assays, the distribution of feeding sites of *A. rosae* larvae was grouped into two categories (see above), and homogeneity of subsets between the four plant treatments was analyzed by χ^2-tests (4×2). Plant treatment effects on time until first feeding of larvae, on egg deposition sites (PI_c), and on numbers of eggs laid by adults were analyzed by one-way ANOVA followed by honestly significant difference (HSD) tests for imbalanced number of subsets. Numbers of eggs within one treatment were examined for differences between both categories by Wilcoxon tests for matched pairs.

Differences of percentages in rhodamin B dye distribution were analyzed by using Wilcoxon tests for matched pairs. To analyze whether weight and dye content of a particular organ were correlated, Pearson–Product moment correlations were executed over all tissues, over only leaves, or only stems.

Results

Induction patterns of plants treated with muslin bags only and nontreated plants only differed in protein concentration and the three myrosinase activity concentrations (soluble, insoluble, and combination of both) in local leaf tissue, which were reduced up to 20% as a result of bag treatment. All other traits in local tissue and each trait measured in systemic tissues were not significantly different between these plant treatments. Bag treatment was the more relevant control compared to damage treatments.

Damage Patterns Within 24 hr, caterpillars of the generalist *S. frugiperda* consumed almost the same amount of leaf tissue as the specialist *A. rosae* larvae but produced a 1.7-fold higher number of damage sites (generalist, 10.5±1.1; specialist, 6.1±0.6; mean±SE, N=8; Mann–Whitney *U* test, Z=−2.6, P=0.020; Fig. 1). Thereby, the total wound length caused by the generalist was nearly twice as long as that caused by the specialist (total wound

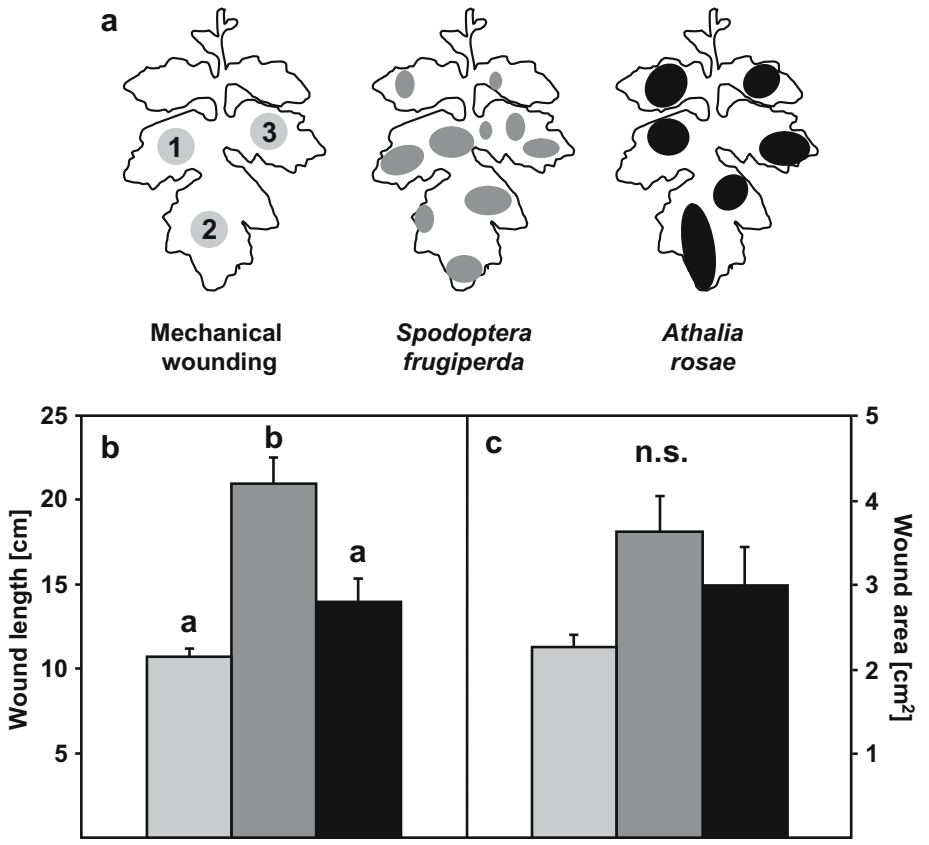


Fig. 1 Damage treatments of *Sinapis alba* leaves: **a** damage patterns after mechanical wounding (three holes at different time points: 10 A.M.; 4 P.M.; 8 A.M., 3 hr before harvest), feeding by *Spodoptera frugiperda* or *Athalia rosae* including mean number of damage sites; wound length (**b**) and removed tissue area (**c**) were determined by photo analysis compared to standards, *letters above bars* indicate significant differences (ANOVA, HSD tests: $P<0.01$, *n.s.* not significant), mean+SE, $N=6–8$

length was calculated as the sum of the perimeters of all damage sites) (ANOVA, $F=16.47$, $df=2$, $P<0.001$; HSD for imbalanced number of subsets $P=0.002$; Fig. 1). Damage by mechanical wounding was similar to feeding by *A. rosae* with respect to wound length and area but not to the number of damage sites that were different (Mann–Whitney *U* test, $Z=-3.1$, $P=0.004$; Fig. 1).

Glucosinolate Concentrations and Myrosinase Activities Eight glucosinolates were detected according to their retention times and UV-spectra in *S. alba* cv. Silenda leaves in decreasing relative amounts: *p*-OH-benzylglucosinolate (85–95%), benzylglucosinolate (5–14%), 4-methoxy-indol-3-ylmethylglucosinolate, indol-3-ylmethylglucosinolate, phenylethylglucosinolate, and three minor putative aromatic glucosinolates not identified. The sum of all six minor compounds represents only about 1% of the sum of all glucosinolates (=total glucosinolates). Capacities of the leaf tissues to hydrolyze glucosinolates were tested as myrosinase activity concentrations against the main glucosinolate *p*-OH-benzylglucosinolate. Total myrosinase activity of a tissue consists of soluble and insoluble fractions that were tested together. Constitutive concentrations of total glucosinolates and total myrosinase

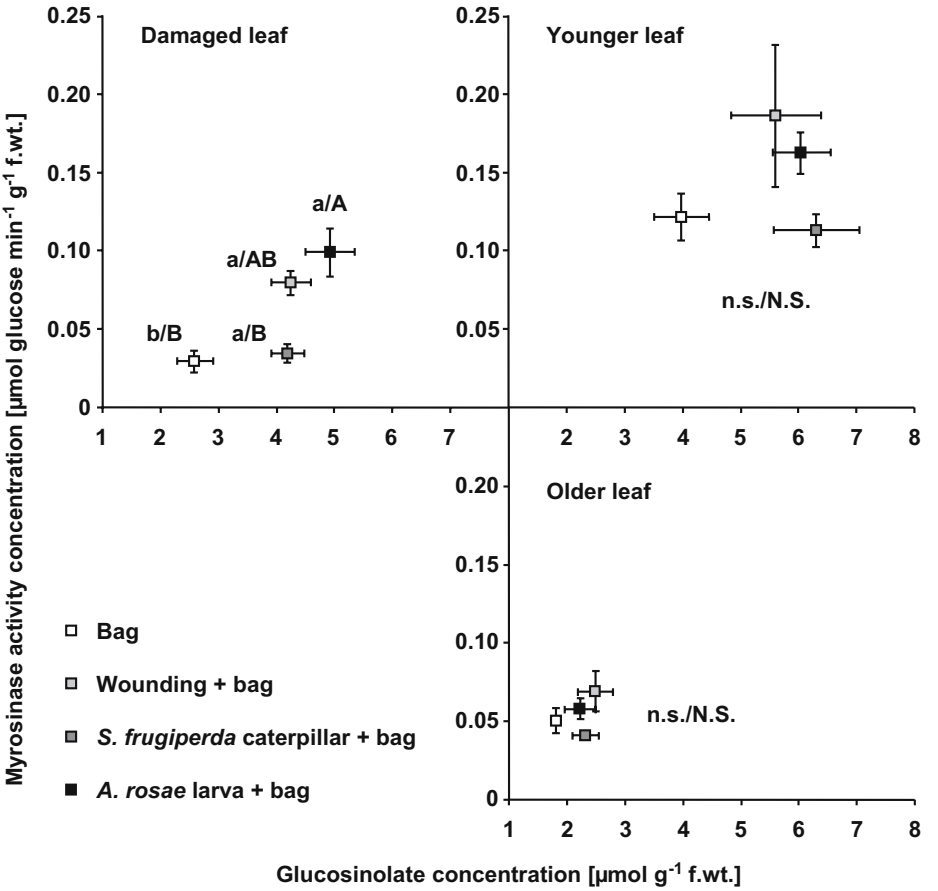


Fig. 2 Correlations of total glucosinolate concentration (sum of eight individual compounds, µmol g^{-1} fw, mean±SE) and total myrosinase activity concentration (µmol glucose min^{-1} g^{-1} fw, mean±SE) in leaves of *Sinapis alba*. The second youngest leaf was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar, or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hr. Bagged leaves without any further treatment served as controls (N=6–8 per treatment). *Letters above bars* indicate significant differences (ANOVA, HSD tests: $P<0.05$, *n.s.* not significant). *Lower case letters* refer to glucosinolate concentrations, *upper case letters* to myrosinase activity concentrations

activity were highest in tissues of younger leaves and decreased more than twofold with leaf age. Upon damage treatment, concentrations of glucosinolates in locally induced leaves increased about 80%, irrespective of inducing agent (Fig. 2). In contrast, significant increases by threefold in concentrations of total myrosinase activity were found in damaged leaves treated with mechanical wounding or specialist feeding only but not in leaves treated with generalist caterpillars. In systemic tissues, increases seemed to be more pronounced in younger than in older leaves, revealing the same patterns as in damaged leaves, but these differences were not significant because of high variation of individual values compared to differences in damaged tissue.

Individual glucosinolates showed different responses (Table 1). Whereas the absolute increase in concentration was highest for the main glucosinolate compound *p*-OH-benzylglucosinolate, indol-3-ylmethylglucosinolate concentrations were increased with the highest relative factor compared to bag-treated controls. In damaged leaves, *p*-OH-

Table 1 Levels of the dominant glucosinolates in leaves of *Sinapis alba*

Organ	Bag	Wounding + bag	*S. frugiperda* + bag	*A. rosae* + bag	ANOVA	
					F	*P*
p-OH-benzyl-gls (μmol g^{-1} f.wt.)						
DL	**2.36** (0.24) a	**3.59** (0.27) ab	**3.66** (0.24) b	**4.55** (0.37) b**	**8.92**	**<0.001**
OL[b]	1.71 (0.07)	2.20 (0.26)	2.07 (0.16)	2.08 (0.23)	0.95	n.s.
YL	3.70 (0.40)	4.98 (0.73)	5.58 (0.65)	5.65 (0.49)	2.35	n.s.
Benzyl-gls ($\times 10^{-1}$ μmol g^{-1} f.wt.)						
DL	2.15 (0.77)	6.12 (2.38)	4.88 (1.15)	3.13 (1.12)	1.33	n.s.
OL[a]	0.91 (0.46)	2.79 (1.07)	2.41 (0.87)	1.30 (0.47)	1.37	n.s.
YL	2.61 (0.90)	5.69 (1.69)	6.74 (1.44)	3.43 (1.10)	2.15	n.s.
Indol-3-ylmethyl-gls (nmol g^{-1} f.wt.)						
DL[a]	**4.09** (1.38) a	**9.94** (2.45) ab	**15.73** (3.47) b	**15.18** (1.98) b	**5.50**	**<0.01**
OL[b]	1.10 (0.37)	1.76 (0.59)	0.87 (0.15)	1.75 (0.42)	1.08	n.s.
YL[b]	7.75 (1.82)	17.65 (5.52)	14.38 (1.63)	14.34 (2.66)	2.03	n.s.
4-Methoxy-indol-3-ylmethyl-gls (nmol g^{-1} f.wt.)						
DL[a]	**13.58** (2.30) a	**14.66** (2.26) a	**19.82** (3.19) ab	**32.02** (5.44) b	**5.30**	**<0.01**
OL[a]	4.27 (0 57)	5.71 (0.96)	6.41 (1.02)	8.61 (1.90)	1.64	n.s.
YL[a]	**13.44** (1.40) a	**18.84** (2.41) ab	**30.80** (6.90) b*	**30.80** (6.08) b*	**3.41**	**<0.05**

The second youngest leaf (DL) was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hr. Bagged leaves without any further treatment served as controls. Mean (SE), N=6–8. Note different scales of single compounds! Treatment effects were tested by one-way ANOVA followed by HSD tests for imbalanced numbers of subsets (significant differences are marked with different letters and values highlighted in bold, $P<0.05$, or otherwise stated).

DL Damaged leaf, *OL* older leaf, *YL* younger leaf; *n.s.* not significant

[a] Data were square root-transformed to ensure variance homogeneity. Back-transformed data means and standard errors are shown.

[b] Data were log-transformed to ensure variance homogeneity. Back-transformed data means and standard errors are shown.

*Differences from control in HSD test: $P<0.08$

**Differences from control in HSD test: $P<0.001$

benzylglucosinolate concentrations increased by a factor of 1.4, 1.6, or 1.9 because of mechanical, generalist, or specialist damage, respectively, compared to the bag control, whereas the corresponding factors for indol-3-ylmethylglucosinolate were 2.4, 3.8, and 3.7. Significant differences of individual glucosinolates compared to control were found only for insect treatments but not for mechanical wounding in damaged leaves. The pattern of benzylglucosinolate induction appeared to be different from the others: In damaged leaves, wounding resulted in the highest increase (13.2±4.6% of total glucosinolates, mean±SE, N=7) although not significant, *S. frugiperda* feeding in intermediate increases (11.3±2.4%), and *A. rosae* feeding had almost no effect (6.0±1.7%) compared to bag treatment (7.4±1.9%).

Overall, increases of soluble myrosinase activity concentrations were low (Fig. 3). In contrast, concentrations of insoluble myrosinase activities increased significantly in damaged leaves compared to controls by a factor of 3.3 or 4.4 as a result of mechanical wounding or specialist feeding, respectively. Generalist feeding had no effect on either soluble or insoluble myrosinase activity concentration in any of the leaves tested.

Soluble Protein and Water Content Protein concentrations were strongly influenced by treatment in damaged leaves (ANOVA, F=4.16, df=3, P=0.016, data were square-root

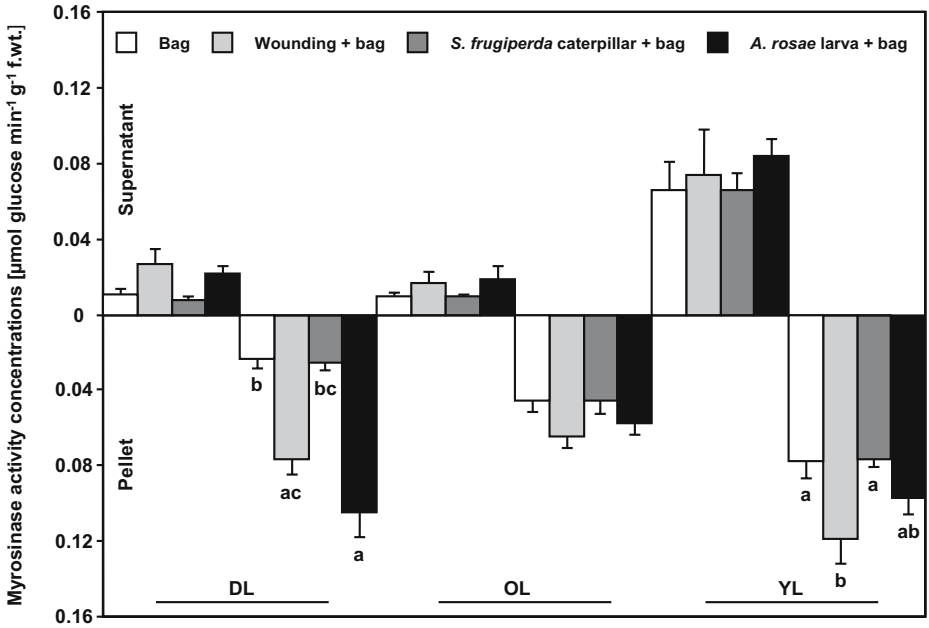


Fig. 3 Levels of myrosinase activity concentrations (µmol glucose min^{-1} g^{-1} fw, mean+SE) of soluble (supernatant, *upper panel*) and insoluble (pellet, *lower panel*) fractions in leaves of *Sinapis alba*. The second youngest leaf was treated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar, or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hr. Bagged leaves without any further treatment served as controls (N=6–8 per treatment). *Letters above bars* indicate significant differences (ANOVA, HSD tests: P<0.05). *DL* Damaged leaf; *OL* older leaf; *YL* younger leaf

transformed to ensure variance homogeneity). Both mechanical wounding (4.9±0.3 mg g^{-1} fresh weight, mean±SE) and feeding by *A. rosae* larvae (4.7±0.9 mg g^{-1} f.wt.) induced soluble protein concentrations by a factor of 1.7 compared to bag treated leaves (2.9± 0.4 mg g^{-1} f.wt.), but *S. frugiperda* caterpillar feeding had no effect (3.3±0.4 mg g^{-1} f.wt.). Differences to controls were significant only for mechanical wounding with P=0.030 (HSD tests). In systemic leaves, *A. rosae* feeding seemed to have stronger effects than the other two damage treatments compared to bag-treated controls, but none of the differences was significant (ANOVA, F=2.38, df=3, P=0.092). Apparent decreases in water content (range, 88.1 to 90.7%) were found only with mechanical wounding, but differences were small and not significant for any organ compared to bag-treated controls.

Behavioral Experiments with Athalia rosae The proportion of larvae feeding on younger tissues in the upper plant region was low in the beginning and increased during the 24-hr test from about 25 to 50% of the larvae feeding in category I. There were no significant differences in attack pattern on differently treated plants at any of the time points tested (χ^2-tests). Time to first feeding as a measure of whole plant acceptance was not significantly affected by plant treatment (ANOVA, F=0.51, df=3, P=0.674).

In the 24-hr test period, each female laid on average 39.2±1.7 eggs (mean±SE, N=52), with no significant differences between plant treatments (ANOVA, F=1.01, df=3, P= 0.397). The mean distribution of eggs laid in categories I and II were similar for all treatments except for mechanical wounding where overall significantly more eggs were laid on older than on younger leaves (Fig. 4). Females did not show any preference for any of

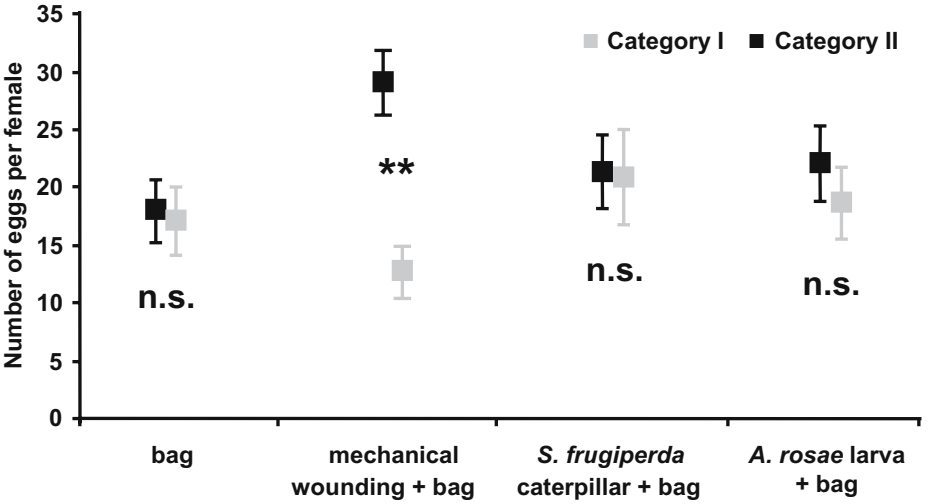


Fig. 4 Number of eggs per female (mean±SE) on category I (induced and younger) and II (all older) leaves of *Sinapis alba* in a 24 hr test. The second youngest leaf was pretreated with mechanical wounding (cork borer), one medium-sized *Spodoptera frugiperda* caterpillar, or one large larva of *Athalia rosae* enclosed in a muslin bag for 24 hr. Bagged leaves without any further treatment served as controls (N=14 per treatment). One female *A. rosae* was enclosed in large muslin bags with one plant for 24 hr. Differences in mean number of eggs per category were tested by Wilcoxon tests for matched pairs (**P<0.01, *n.s.* not significant)

these categories within plants treated with bags only (PI_c=−0.05±0.14, mean±SE, N=14; Wilcoxon test, Z=0.38, P=0.706), with *S. frugiperda* caterpillars (PI_c=−0.07±0.12, N=14; Wilcoxon test, Z=0.06, P=0.950), or *A. rosae* larvae (PI_c=−0.08±0.12, N=10; Wilcoxon test, Z=0.30, P=0.767). However, a clear preference for category II leaves was found in plants treated with mechanical wounding (PI_c=−0.39±0.16, N=14; Wilcoxon test, Z=2.76, P=0.006). No significant effects because of treatment of plants were found for PI_c of individual females (ANOVA, F=1.87, df=3, P=0.147).

Plant Vascular Connectivity Rhodamin B content was higher in leaves than in stems. Signals of stem tissue samples were below the detection limit in many cases. The recovery rate of applied dye was 81±6% (mean±SE, N=9). Some of the dye was incorporated into the hydrophobic sealing grease and could not be solubilized by nanopure water. The distribution of dye among the five organs was 35.1±2.7% (mean±SE, N=9) in treated leaves, 40.7±1.8% in younger leaves, 19.4±2.8% in older leaves, 4.5±0.6% in younger stems, and in older stems 7.5±1.3% of the recovered amount of rhodamin B per plant. The dye accumulated in treated and younger leaves, with significant differences between older leaves and treated leaves (Wilcoxon test, Z=2.07, P=0.038) and between older and younger leaves (Wilcoxon test, Z=2.54, P=0.011). We found an overall correlation between organ fresh weight and accumulated dye per organ within all tissues (Pearson's correlation coefficient, R^2=0.29, $F_{1,32}$=13.6, P<0.001), but not within leaves or stems only.

Discussion

Specificity According to Mode of Induction Plants of *S. alba* cv. Silenda treated with mechanical wounding, generalist, or specialist feeding induced total glucosinolate

concentrations to a similar extent, yet their composition varied. Mechanical wounding and feeding by *A. rosae* larvae on *S. alba* leaves induced increases in protein and myrosinase activity concentrations mostly to the same extent, but no significant increase in those parameters could be found in leaves damaged by *S. frugiperda* (Fig. 2). Plant responses to different inducing agents roughly reflect the differences in wounding patterns. Damage patterns (Fig. 1) after mechanical wounding and specialist *A. rosae* feeding were similar, but damage inflicted by generalist caterpillars of *S. frugiperda* resulted in about twice longer wound perimeters, a measure for dose of damage.

A dose-dependency of damage was shown previously for the induction of indolic and aliphatic glucosinolates upon mechanical wounding (Koritsas et al. 1991; Bodnaryk 1992) and upon application of the wound-induced plant hormone jasmonic acid (Bodnaryk 1992; Doughty et al. 1995). Probably, dose-dependent influences also exist for induction of aromatic glucosinolate concentrations or myrosinase activities in *S. alba*. Many plant responses, however, are dependent on the concentration, place, and mode of action of elicitors present in saliva or oviposition secretion of a given herbivore (Felton and Eichenseer 1999; Hilker and Meiners 2006 and references therein). Salivary compounds do not always elicit an increase of metabolites. Glucose oxidase or pH-effects that influence methanol release were shown to inhibit plant responses that otherwise occur upon mechanical wounding (Musser et al. 2005; Bede et al. 2006; von Dahl et al. 2006). We did not detect glucose oxidase activity in head or body extracts of *S. frugiperda* nor in extracts of *A. rosae* by using the method described by Eichenseer et al. (1999) (data not shown). Other elicitors, i.e., fatty acid-amides, have also been reported for caterpillars of *S. frugiperda* (Spiteller and Boland 2003). Additionally, the temporal pattern of wounding can be important for induction, as has been shown for the release of volatiles in lima beans as result of feeding by *Spodoptera littoralis* (Boisd.) (Mithöfer et al. 2005). We applied mechanical damage three times, interspaced as to mimic not only spatial but also broad-scale temporal pattern of herbivore feeding. Nevertheless, we are aware that herbivore feeding differed from our mechanical treatment because plant tissue was removed in small bits by the herbivores but in larger bulks by cork borer wounding.

Within an individual plant, the stronger response to damage in treated and younger systemic tissue compared to older systemic tissue in *S. alba* cv. Silenda can be explained by the tissue connectivity that was visualized by the transport of the dye rhodamin B. The amount of dye recovered after 24 hr was about twice higher in treated and younger than in older leaves. The relocation of dye among leaves was not correlated to organ biomass and is thought to be a result of source–sink relationships modulating bulk phloem flows (Orians 2005). Similar relationships of induction responses among differently aged leaves have also been found in other systems (Orians et al. 2000; Viswanathan and Thaler 2004). The differences in strength of response between local and systemic younger leaf tissues might be based on a "signal" delay in systemic tissue because of transportation processes.

Specificity According to Plant Cultivar Variation in quantity and quality of plant defenses is known to occur among species, individual plants, and different organs (Brown et al. 2003; Charron et al. 2005). Crop production, for which a diversity of cultivars have been bred, adds another level of specificity (for *S. alba* see: Hopkins et al. 1998a, b; Schuster-Gajzágó et al. 2006). However, few studies have compared these cultivars with respect to induction properties of glucosinolates and even fewer to those of myrosinase activity (Ludwig-Müller et al. 1997; Hopkins et al. 1998a, b; Andréasson et al. 2001). The *S. alba* cultivar Silenda used in this study and the *S. alba* cultivar Salvo previously investigated (Martin and Müller 2007) differed not only with respect to constitutive glucosinolate and myrosinase activity

concentrations but also with respect to induction responses to feeding by larvae of *A. rosae*. Upon induction with 6-d-old larvae, local responses in damaged leaves were stronger in the cultivar Silenda than in Salvo for total glucosinolate concentrations as well as for all three myrosinase activity concentrations (soluble, insoluble, and combination of both). The systemic response of glucosinolate concentrations was stronger in the cultivar Silenda, but changes of myrosinase activity concentrations were stronger in leaves of the cultivar Salvo. Thus, we showed that specificity of short-term induction response upon feeding by a glucosinolate-sequestering specialist herbivore varies strongly with respect to cultivar, as has also been shown for studies of long-term induction effects of the nonsequestering specialist *Delia floralis* (Fall.) (Hopkins et al. 1998a, b) and of pathogens (Ludwig-Müller et al. 1997; Andréasson et al. 2001) in other Brassicaceae.

Consequences for Interaction The defensive properties of differentially induced plants and their acceptability were tested against subsequent attacks of *A. rosae* larvae and egg-laying females. Larvae usually prefer to move to the upper plant parts (personal observation). When placed at the bottom of a plant pot, movement of larval feeding pattern was comparable on all differently pretreated plants. Thus, larvae seem to be unaffected by different plant induction states, specifically in the upper plant region where changes are more pronounced. Females distributed their eggs similarly on young and old plant parts in most plant treatments (Fig. 4). However, marginal behavioral modifications were observed in response to mechanically wounded plants, where females laid fewer eggs on damaged and younger systemic than on older leaves. These plants showed differences in glucosinolate profiles, especially in benzylglucosinolate concentrations, compared to the other treatments. Females of *A. rosae* responded differently to isothiocyanates, the hydrolysis products of glucosinolates, with varying side-chains (Barker et al. 2006), but it is not clear whether altered glucosinolate profiles in *S. alba* might have influenced choices for egg deposition sites in our bioassays. In other systems, side chain modifications of glucosinolates led to modulations in disease resistance (Brader et al. 2006) and in specialist feeding damage (Giamoustaris and Mithen 1995) but not in generalist feeding and performance (Giamoustaris and Mithen 1995; Burow et al. 2006). Overall, the observed induction responses in *S. alba* did not serve as rapid plant protection against *A. rosae* larvae or egg-laying females, regardless of the inducing agent. However, long-term induction responses were not investigated in this study, and such studies might reveal different aspects of resistance against a sequestering herbivore in the field. In a former study, almost no differences were found in the long-term development of larvae and adults of *A. rosae* raised on *Brassica juncea* lines with differing glucosinolate and myrosinase contents (Müller and Sieling 2006). In contrast, other nonsequestering specialists have been found to have a reduced performance in long-term (Agrawal 2000) and short-term trials (de Vos et al. 2006) on induced plants. The glucosinolate-sequestering herbivore *A. rosae* thus seems highly tolerant to variation in the glucosinolate–myrosinase system evoked either by rapid induction changes or by permanent modifications in different inbred lines.

Induction responses in *S. alba* do not seem to benefit the plant, but rather its opponents: The sequestering specialist might profit from increased glucosinolate levels and thus increased protection because of sequestration. The generalist herbivore suppresses induction responses of hydrolyzing enzymes and might obtain less toxic food this way. Therefore, in *S. alba*, the lethal plant defense paradox (Fordyce 2001) is present for the cultivar Silenda, as increased glucosinolates means benefits for the sequestering herbivore (Müller et al. 2002). The selective increase, as a result of *A. rosae* feeding, of myrosinase activity concentrations only in systemic leaves of the cultivar Salvo (Martin and Müller 2007) might have a

protective function for the plant. In *B. juncea*, *A. rosae* larval feeding reduced glucosinolate and myrosinase activity levels within 24 hr (Müller and Sieling 2006). This suggests that not only has the lethal plant defense paradox been solved to different extents in plants but also that there is no universal short-term response of plants to one herbivore.

The induction responses found for the glucosinolate–myrosinase system in *S. alba* showed some specificity because of the inducing agent. Glucosinolates and myrosinases revealed different induction patterns pointing at separate regulation mechanisms. Insect-derived elicitors and the redistribution of a potential signal via the phloem seem likely to be involved in this specificity. Nevertheless, these plant differences were not mirrored in behavioral responses of larvae or adult females of *A. rosae*.

Acknowledgments The authors thank M. Riederer for making lab space and HPLC equipment available, D. Imes and D. Paltian for help with numerous extractions and the Bayer AG for *Spodoptera frugiperda* eggs. This research was supported by the Sonderforschungsbereich 567 "Mechanismen der interspezifischen Interaktion von Organismen" of the Deutsche Forschungsgemeinschaft.

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triterpenoid saponins. Some authors also distinguish a third group, the so-called steroidal amines that can be classified as steroidal alkaloids (Bruneton 1995). Steroidal saponins consist of a steroidal aglycone, a C_{27} spirostane, generally composed of a six-ring structure (Fig. 1a). Within fresh plant tissues, the hydroxyl group in the 26-position participates in a glycosidic linkage, and the aglycone structure remains as pentacyclic furostane (Fig. 1b). Triterpenoid saponins consist of a triterpenoid aglycone composed of a C_{30} pentacyclic structure (Fig. 1c; Sparg et al. 2004).

Saponin concentrations in different alfalfa varieties have been reported and range from 0.8–2% (Pedersen and Wang 1971; Majak et al. 1980). Pecetti et al. (2006) also showed saponin differences among alfalfa cultivars with compounds such as medicagenic acid and zanhic acid.

Saponins are toxic to herbivorous insects (Ishaaya et al. 1969; Shany et al. 1970; Oleszek et al. 1992), are reported to be important resistance factors to the pea aphid, and have been suggested a potentially useful for alfalfa resistance breeding programs (Horber et al. 1974). High saponin content in some alfalfa cultivars is related to resistance against the pea aphid, *Acyrthosiphon pisum* (Pedersen et al. 1976). Saponins have also been shown to interfere with aphid feeding behavior (Golawska et al. 2006).

In this paper, the effects of isolated alfalfa saponins on pea aphid feeding behavior are examined in detail. Three major alfalfa saponins—(**1**) 3GlcA, 28AraRhaXyl medicagenic acid glycoside, (**2**) 3GlcA, 28AraRha medicagenic acid glycoside, and (**3**) zanhic acid tridesmoside—were used in *in vitro* experiments. Saponin effects on aphid probing of the plant peripheral tissues and phloem phase were studied.

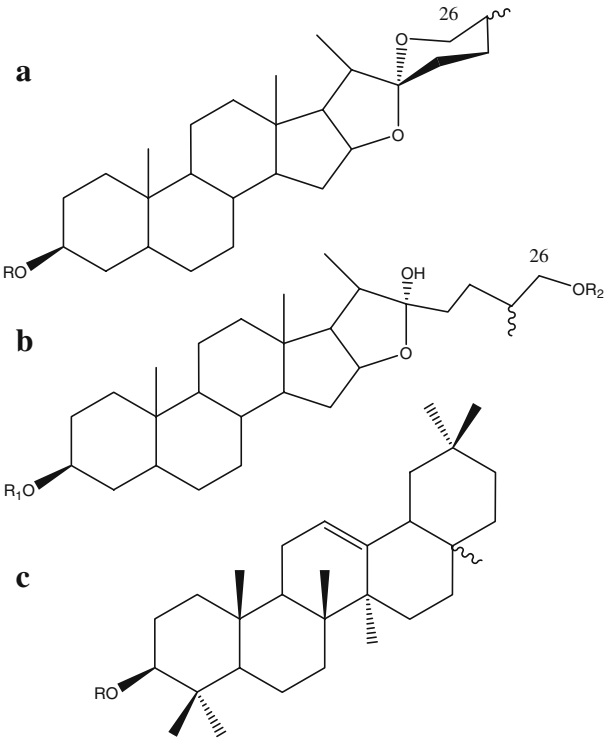


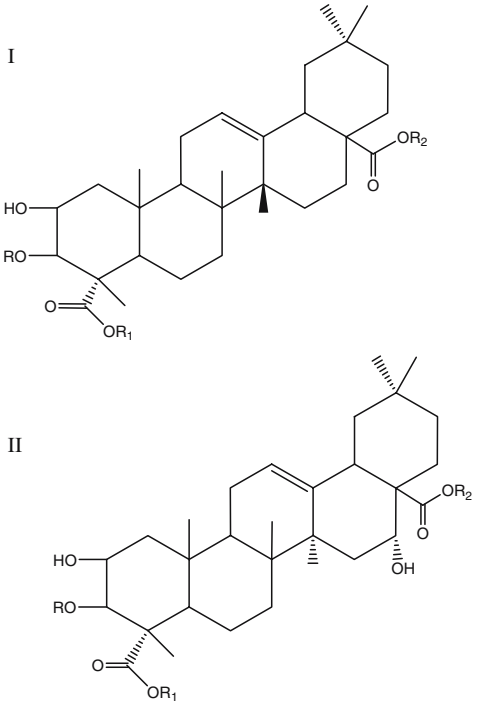
Fig. 1 Aglycone frames of saponins: **a** steroidal spirostane; **b** steroidal furostane; and **c** triterpenoid saponins. *R* Sugar moiety

Methods and Materials

Aphid Culture The pea aphid, *A. pisum* Harris, wingless females were used in all feeding behavior experiments and came from a stock culture kept at the University of Podlasie, Siedlce, Poland. Aphids were collected from a laboratory culture reared on broad been (*Vicia faba* L. var. Start) and maintained in an environmental chamber at about 21°C, L16:D8 photoperiod, and 70% RH.

Chemicals Individual saponins were obtained from the Institute of Soil Science and Plant Cultivation, Pulawy, Poland. Compounds **1**, **2** and **3** (Fig. 2) were isolated from alfalfa as described by Oleszek et al. (1990).

Application of Tested Compounds The effect of saponins on pea aphid feeding behavior was investigated *in vitro*, using sucrose–agarose gels. Control gels (without saponins) were prepared by incorporating 1.25% agarose (Sigma A-0169) into 30% sucrose solution. Experimental gels (containing saponins) were prepared by incorporating individual saponins at concentrations of 50 or 100 ppm. After stirring, the mixtures were heated in



Compound	Aglycon	R	R_1	R_2
1	I	GlcA	H	-Ara-Rha-Xyl
2	II	GlcA	H	-Ara-Rha
3	III	-Glc-Glc-Glc-	-Ara-	-Ara-Rha-Xyl-Api

Fig. 2 Structures of compounds 1–3

a water bath (75°C for 30 min) and then poured into plastic rings covered by a stretched Parafilm M® membrane. Transparent gels formed after 1–2 min and offered to aphids for probing.

Electrical Penetration Graph Recordings The probing behavior of adult apterous aphids was recorded using a DC electrical penetration graph (EPG) amplifier (type Giga-4). Aphids were connected to the EPG system by a 2-cm gold wire (20 μm in diameter) and attached with conductive silver point (Demetron, L2027, Darmstadt, Germany). A second electrode was introduced into the gel. EPG recordings were performed inside a Faraday cage under laboratory conditions (ca 24°C for 4 hr). Collected insects were starved for 2 hr and then placed onto gels. Pea aphid probing behavior was recorded as follows: One adult apterous aphid was placed onto each gel and EPG recordings were made for 10 aphids on 10 different gels without saponins (control) and 10 gels for each concentration of the tested compounds. Aphid feeding behavior was monitored for 4 hr.

EPG Analysis Acquisition and analysis of EPG signals were done with STYLET 2.2 software (ref). Waveform patterns were identified according to Tjallingii (1990). Insect feeding behavior was based on aphid activities and noted as follows: non-probing (np pattern, aphids cannot start probing), path (C pattern—pathway; penetration of peripheral tissues—epidermis and mesophyll), salivation into sieve elements (E1 pattern), ingestion of phloem sap (E2 pattern—aphid feeding), and ingestion of xylem sap (G pattern). EPG parameters were measured in each group and recalculated per one insect. Data obtained with experimental and control insects were subjected to one-way analysis of variance followed by Duncan's test.

Results

Saponins clearly affect feeding behavior of *A. pisum*. EPG recordings indicated that the pea aphid behaved in a variable manner while probing artificial diets containing test compounds. On each occasion, aphid-probing activity was composed of major EPG patterns: pathways (C), sieve element salivation (E1), phloem sap ingestion (E2), and xylem sap ingestion (G) (Table 1). Among the saponins studied, **2** and **3** were more

Table 1 Probing behavior of *A. pisum* on artificial diets

Variant	Concentration of Saponin (ppm)	EPG Parameters[a]
Control	None	C, E1, E2, G
1	50	C, E1, E2, G
	100	C, E1, E2, G
2	50	C, E1, E2
	100	C, E1, E2
3	50	C, E1, E2,G
	100	C, E1, E2

[a] C—EPG waveforms A, B and C reflecting intercellular stylet penetration activities; E1 and E2—EPG waveforms reflecting salivation into and ingestion of phloem sap; G—EPG waveforms reflecting ingestion of xylem sap

Table 2 Effect of different concentrations of saponins on probing behavior of *A. pisum*

Variant	Concentration (ppm)	Number of Penetrations	Time of the First Probing (min)	Average Time of Probing
Control	None	48.0a±1.05	0.725b±0.018	7.063a±0.018
1	50	43.3a±8.74	1.290b±1.430	1.235c±0.360
	100	26.2b±13.40	1.757b±1.630	2.358bc±1.511
2	50	25.3b±14.53	0.847b±0.792	1.657bc±0.912
	100	23.2b±8.01	1.120b±0.377	3.105bc±1.589
3	50	20.8b±6.60	0.977b±0.876	1.440bc±0.925
	100	11.4b±1.84	5.500a±3,103	4.124b±1.235

Values in columns not followed by the same letters are significantly different at $P<0.05$ (Duncan's test).

effective modulators of probing behavior than **1**. The total number of gel penetrations by the pea aphids and duration of the pathways were reduced by higher concentrations of compounds (Table 2). Compounds **1** (at 100 ppm), **2** (at both 50 and 100 ppm), and **3** (at 50 ppm) reduced the number of gel penetrations by approximately half. At 100 ppm, compound **3** decreased the number of penetrations by four times (Table 2). Saponins also prolonged the first probe by experimental aphids compared to controls—about twice the duration for saponins **1** and **2** at concentrations of 50 and 100 ppm, and over seven times in the case of saponin **3** at 100 ppm (Table 2). For all tested saponins, reduction in average duration of probing activity in relation to control gels was observed (Table 2).

A similar tendency was found for aphid salivation into sieve elements and phloem sap ingestion (Table 3). At a concentration of 50 ppm, all saponins reduced duration until the first E1 pattern and for 3GlcA, 28AraRhaXyl medicagenic acid up to seven times. Prolongation of such aphid activity was observed for **1** and **2** at a concentration of 100 ppm and **3** at 50 ppm. Generally, the tested concentrations of **2** and **3** reduced total time of pea aphid salivation into the gels (Table 3). Saponins also modified the duration of the first

Table 3 Effect of different concentrations of saponins on feeding behavior of *A. pisum* at phloem phase during probing of artificial diets

Aphid activity (min)	Control	Concentration of Saponins					
		50 ppm			100 ppm		
		1	2	3	1	2	3
Time Until First E1 Pattern	52.090ab± 0.176	7.100b± 9.091	39.913ab± 30.348	44.327ab± 67.144	82.610a± 54.4181	31.983ab± 54.884	15.970ab± 33.668
Time the First E1 Pattern	1.302a± 0.022	0.540a± 0.773	2.917a± 2.638	9.033a± 17.289	4.833a± 5.0465	12.603a± 24.021	0.420a± 0.886
Total Time of E1 Pattern	38.333ab± 0.458	38.340ab± 32.067	16.530bc± 9.932	24.330abc± 36.406	51.773a± 37.0637	19.473abc± 22.911	3.200c± 6.746
Time Until First E2 Pattern	30.000b± 0.458	2.283b± 1.973	116.213a± 76.513	44.780ab± 73.344	42.860ab± 56.6820	5.190b± 10.942	16.386b± 34.545
Time the First E2 Pattern	1.333ab± 0.042	2.243a± 1.935	0.630ab± 0.607	1.207ab± 1.516	1.390ab± 1.8191	0.453b± 0.956	0.094b± 0.198
Total Time of E2 Pattern	2.000b± 0.243	26.017a± 22.397	5.223b± 7.975	22.480a± 22.786	4.970b± 6.9424	1.293b± 2.727	0.234bc± 0.493

Means in rows followed by the same letters are not significantly different at $P<0.05$ (Duncan's test)

phloem sap ingestion (Table 3). In addition, the total time of this aphid activity was prolonged in the presence of all 3 saponins at 50 ppm. Higher concentrations (100 ppm) reduced the duration of phloem sap ingestion except for **1** (Table 3).

Compound **1** did not affect xylem sap ingestion at the tested concentrations (Table 4). When **2** was added to gels, pea aphid activity corresponding to xylem sap ingestion did not occur (Table 4). At the lower concentration, compound **3** reduced this phase of aphid feeding behavior and at the higher concentration completely stopped this activity.

Discussion

The EPG recordings of pea aphid probing of sucrose–agarose gels containing different concentrations of saponins showed clear differences in feeding behavior. Generally, higher concentrations caused reduction of aphid activities that corresponded to ingestion of phloem and xylem sap. Similar findings have been reported previously for saponins isolated from seeds of *Barringtonia asiatica* in relation to *Epilachna* sp. larvae (Herlt et al. 2002). EPG results indicated moreover that the pea aphid avoided ingesting saponins. The concentrations of saponins used in this study corresponded to levels within a low-saponin line of alfalfa and were lower than those found in high-saponin lines of the Radius cultivar (Staszewski et al. 1994). Thus, the antibiotic effect of alfalfa saponins on pea aphid might be even stronger in a high-saponin line.

Synergism among the tested saponins was not studied in these experiments; however, synergistic interactions among saponins may occur within alfalfa tissues. Horber et al. (1974) and Adel et al. (2000) reported that saponins may act synergistically. Moreover, they may also act synergistically with other groups of alfalfa allelochemicals as reported for other plants and insects (Sutherland et al. 1982; Berenbaum 1985).

The results here obtained did not confirm earlier reports by Oleszek et al. (1992). The tested compounds may be antifeedant compounds for pea aphid—Fabaceae relationships. Szynkarczyk et al. (2000) showed that *A. pisum* fed on high-saponin alfalfa lines reduced pea aphid performance and phloem sap ingestion. Golawska et al. (2006) further showed differences in pea aphid feeding behavior on alfalfa with low and high saponin content. Aphids fed on high-saponin lines had prolonged penetration of the epidermis and mesophyll (pattern C) and showed a significant reduction in phloem sap ingestion. On

Table 4 Effect of different concentrations of saponins on feeding behavior of *A. pisum* at xylem phase during probing of artificial diets

Aphid activity (min)	Control	Concentrations of Saponins					
		50 ppm			100 ppm		
		1	2	3	1	2	3
Time Until First G Pattern	148.667a± 0.2671	10.527b± 9.066	0.00c± 0.000	4.160b± 4.113	59.027b± 85.778	0.00c± 0.000	0.00c± 0.000
Time the First G Pattern	26.667a± 0.6722	0.283b± 0.246	0.00b± 0.000	15.830ab± 19.838	13.523ab± 16.109	0.00b± 0.000	0.00b± 0.000
Total Time of G Pattern	35.833ab± 0.2137	44.587a± 38.380	0.00c± 0.000	35.687ab± 37.411	34.160ab± 36.630	0.00c± 0.000	0.00c± 0.000

Values in rows followed by various letters are significantly different at $P<0.05$ (Duncan's test)

the other hand, negative effects of saponins on herbivore performance, e.g., reduction of growth and pupal mass, could be a consequence of shortening or suppressing the feeding process. Thus, the results presented here confirm that alfalfa saponins are natural feeding barriers for phytophagous insects (Sutherland et al. 1982; Meisner and Mitchell 1983; Potter and Kimmerer 1989; Jain and Tripathi 1991; Nozzolillo et al. 1997). Agrell et al. (2003) demonstrated that alfalfa showed an herbivore-induced resistance based on saponins and that such induction affected feeding behavior and exerted negative effects on herbivore physiology. We documented that saponin *3* is important in chemical interactions between host-plants and herbivores. Moreover, a similar reduction in insect food consumption by saponins has been reported by Adel et al. (2000). They were the major cause of diminished body growth and possibly extension of the feeding period. Alfalfa saponins administered to larval *Spodoptera littoralis* through diet caused prolongation of both larval and pupal stages, retarded growth, increased mortality, and reduced fecundity and fertility. There are data also that indicate that saponins slow the passage of food through the gut, perhaps by reducing digestibility, and may secondarily influence food uptake. Inhibition of digestive enzymes (Ishaaya and Birk 1965) and interference with sterol metabolism (Ishaaya et al. 1969; Shany et al. 1970) also may be involved with the effects of alfalfa saponins on food processing in *S. littoralis.* In addition, extension of pupal instars and reduction of adult fertility have been reported by Hubrecht et al. (1989). Szynkarczyk et al. (2001) and Golawska et al. (2005) found a negative relationship between saponin concentration and development of pea aphids on alfalfa lines. Horber (1972) used commercial saponins isolated from alfalfa and found that even low concentrations may exert an adverse effect on the pea aphid. Finally, there are reports of the high toxicity of saponins to other insects (Bondi and Birk 1968; Applebaum et al. 1969; Thorp and Briggs 1972; Sutherland et al. 1975a, b).

The current studies suggest that saponins *1*, *2*, and *3* are toxic toward the pea aphid and may have potential as alfalfa resistance factors toward the pea aphid. Thus, it might be worthwhile to modify saponin levels in the breeding of modern alfalfa lines with biotechnologically introduced genes that regulate saponin concentration and composition.

Acknowledgments I thank Prof. W. Oleszek (Institute of Soil Science and Plant Cultivation, Pulawy, Poland) for providing samples of individual saponins.

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photobiont. The fungal partner often produces so-called lichen substances, i.e., secondary acetone-extractable metabolites (Huneck and Yoshimura 1996). These are chemically diverse compounds, generally biosynthesized through the acetate–mevalonate pathway, but some through the shikimic acid pathway (pulvinic acid derivatives). Brown melanic compounds are complex and poorly known polymers of phenolic compounds (recently reviewed for microorganisms including fungi by Plonka and Grabacka 2006). They cannot be extracted, but they can be quantified by spectral reflectance measurements.

Most lichen substances, including melanic compounds, strongly absorb UV-B radiation, and colored compounds additionally absorb some visible radiation. Some compounds are located in the upper cortex of the lichen thallus (e.g., usnic acid, parietin, and melanic compounds), and may screen the underlying algal cells from excess UV-B and/or visible radiation (Ertl 1951; Büdel and Lange 1994; Dietz et al. 2000; Gauslaa and Solhaug 2001; Solhaug et al. 2003; Nybakken et al. 2004). Variations in thallus color and contents of cortical substances between sun and shade forms of some lichen species were acknowledged early and interpreted as acclimation to different light regimes (Richardson 1967; Rundel 1969). Many medullary substances, often located within the photobiont level, are assumed to screen excess solar radiation because of their strong UV-B absorbance. In addition, both medullary and cortical lichen substances function as herbivore defense (Slansky 1979; Lawrey 1983; Gauslaa 2005; Nimis and Skert 2006), allelopathics (Pyatt 1967; Brown and Mikola 1974; Fisher 1979), and/or antibiotics (Gudjónsdóttir and Ingólfsdóttir 1997; Cocchietto et al. 2002). Such functional roles imply that a species' success, in some ways, depends on the concentration and composition of lichen substances.

In a previous study, we assessed growth in three epiphytic old forest lichens (the green-algal *Usnea longissima*, the cyanobacterial *Pseudocyphellaria crocata*, and the tripartite *Lobaria pulmonaria*) transplanted into three successional stands in boreal spruce forests (dense young forests, open old forests, and clear-cuts) at three locations along a rainfall gradient (Atlantic, suboceanic, and continental climatic zones) (Gauslaa et al. in press). The low light in the young forests severely restricted growth of all three species, and for the alectorioid *U. longissima*, low light limited growth even in the open old forests. The two foliose species grew best in old forests, and showed some damage in terms of chlorophyll degradation because of excess light in the sun-exposed clear-cuts. For all species, growth was substantially higher in the Atlantic rainforest compared with the drier suboceanic and continental locations.

As part of a search for mechanisms behind the reported variation in acclimation traits for the three studied lichens (Gauslaa et al. in press), an objective was to assess possible successional stand-specific differences in secondary chemistry. Lichen substances with functional roles in sun protection are expected to increase along the light gradient. Both *L. pulmonaria* (Gauslaa and Solhaug 2001) and *P. crocata* can produce cortical melanic, nonextractable pigments, which may absorb substantial quantities of visible light in addition to UV-B (Nybakken et al. 2004). The two melanic species showed signs of damage in the exposed clear-cuts during the transplantation (Gauslaa et al. in press), presumably because of excess temperatures (Thus, according to the results of Gauslaa et al., we expected the most successful cortical screen to be found in the third species, *U. longissima*, which showed no signs of photodamage and even had the highest growth on the clear-cuts.).

Methods and Materials

Lichen Material All lichens were collected in boreal *Picea abies*-dominated forests. *Lobaria pulmonaria* (L.) Hoffm. was collected in rainforests in Overhalla (64°27′N 11°53′E,

15 m asl) and Namsos (64°25′N 11°25′E, 20 m asl) in Nord-Trøndelag, Norway from twigs of *P. abies* and scattered stems of *Salix caprea*. *Usnea longissima* Ach. was collected from lower branches of spruce trees in steep, NE-facing open and old forests in Toten (60° 35′N, 11° 02′E, 600–700 m asl), Oppland, E Norway. *Pseudocyphellaria crocata* (L.) Vain. was collected in two locations in Namsos (64°25′N 11°25′E, 20 m asl and 64°25′N 11°25′E, 40 m asl) and one in Namdalseid (64°20′N 11°17′E, 40 m asl). All thalli were air-dried in the lab at the collection day. During the approximately 1-mo period between collection and transplantation, thalli were stored air-dry in a fridge. Further details are given in Gauslaa et al. (in press).

Study Sites Three *P. abies*-dominated transplantation locations were chosen: an Atlantic location with coastal rainforest in Namsos, Nord-Trøndelag, W Norway (64°25′N 11°27′E, 30 m asl); a suboceanic location in Siljan, Telemark, SE Norway (59°22′N 9°45′E, 500 m asl); and a continental location in Kullbäcksliden, near Umeå, Västerbotten, N Sweden (64° 10′N 19°40′E, 250 m asl). Total mean daily temperatures were 12–13°C in all three areas during the transplantation period. The rainfall during the transplantation period varied between 440 mm (81 rainy days) in the Atlantic location and 276 mm (54 rainy days) in the continental location, with the suboceanic location (309 mm; 43 rainy days) in between. *Lobaria pulmonaria* and *U. longissima* were transplanted to all three sites because they are native to all three respective climatic zones. The rainforest lichen *P. crocata* was only transplanted to the Atlantic zone to which it is restricted. In the present study, the two foliose lichens *P. crocata* and *L. pulmonaria* were measured from the Atlantic site only, whereas the most responsive species in terms of growth (Gauslaa et al. in press), *U. longissima*, was measured from all three climatic zones.

Transplantation Experiment Before and after the transplantation period, the hydrated thallus area was measured with a leaf area meter (LI3100 Li-Cor, Lincoln, NE, USA). Thereafter, lichens were desiccated 48 hr at 20°C before recording the dry weight. Four randomly chosen thalli of *L. pulmonaria* were fastened with a thin thread onto nylon net on each of 45 square frames (15.9×15.9 cm). Because of limited material of *P. crocata*, only two to three thalli were fastened in the same way on each of 45 new frames. Lobes of these two foliose lichens were positioned horizontally. The position simulates the typical orientation of the lichens on *P. abies* where they are restricted to branches and thin twigs. Each of the pendulous *U. longissima* thalli was fastened with a thin thread through small holes to one of four vertical sticks on frames similar to those used for the foliose lichens. The sticks allowed a vertical hanging position of pendulous thalli. The lower lichen parts were hanging freely above the horizontally located frames. Frames with *U. longissima* were placed in all three sites, 45 frames on each, on a horizontal frame holder on a stick 1 m above the ground. Fifteen frames with each lichen species were randomly placed in each of three successional stands at each transplantation location: (1) even-aged young and closed canopy stand, mean indirect site factor (ISF) according to Anderson (1964): 0.141–0.224; (2) old forest with gaps, ISF: 0.229–0.278; and (3) open clear-cut areas ISF: 0.647–0.840. The ISF is the proportion of diffuse solar radiation reaching a given location, relative to that in a location with no sky obstructions (see Gauslaa et al. in press for description of measurements of ISF). Transplants were kept in the field from June 11 to September 30 (the Atlantic site; 110 d, 81 rainy days with 440 mm rainfall), June 13 to October 1 (the suboceanic site; 111 d, 43 rainy days with 309 mm), and June 18 to October 4 (continental site; 111 d, 54 rainy days with 276 mm), all in year 2005. After harvest, the transplants were transported to the lab the same day and left to dry at room temperature.

Extraction of Lichen Compounds Preliminary experiments showed that the secondary compounds were extracted completely from the thin alectorioid *U. longissima* by rinsing intact air-dry thalli in pure acetone. Two thalli from each frame (30 thalli per successional stage at each site) were put into 10-ml glass tubes and 5–7 ml of acetone was added. After 20 min of extraction, the acetone was poured into 30-ml glass tubes, and new acetone was added to the thalli. The extraction was repeated three times, the four supernatants combined, and the acetone was left to evaporate from the glass tubes in a 50°C water bath in a fume cap. Dried extracts were stored dark and dry for a maximum of 24 hr before they were dissolved in 10 ml of acetone and run on high-performance liquid chromatography (HPLC).

Preliminary tests for the thick foliose *L. pulmonaria* and *P. crocata* showed that the secondary compounds were extracted most efficiently from hydrated thalli immersed in *N*, *N*-dimethylformamide (DMF). Two thalli from each frame (30 thalli per successional stage) were cut into two equal parts, one was randomly selected, and weight and area were measured. The selected part was then put in 10 (*L. pulmonaria*) or 5 (*P. crocata*) ml of DMF and left for 5 d in a dark fridge before the DMF extract was run on HPLC.

HPLC Analysis Lichen extracts were analyzed on a 1100 Series HPLC (Agilent Technologies, Waldbronn, Germany) including a 1,040-M diodearraydetector. Separation was achieved on an ODS Hypersil 60×4.6 mm column. Oven and injector temperatures were 25 and 22°C, respectively. The injection volume was 20 μl for DMF extracts and 10 μl for acetone extracts, and the flow rate was 2 ml min^{-1}. Solvent A consisted of 0.25% orthophosphoric acid and 1.5% tetrahydrofuran in Millipore water, whereas solvent B was 100% methanol. The run started with 30% B. Within 15 min, solvent B was increased to 70% and further to 100% the next 15 min, and then isocratically in 100% B for a further 5 min. At the end of the run, solvent B was reduced to 30% within 1 min, and the column flushed with 30% B for 5 min before the next run started.

The detection wavelength was 245 nm, and the identification of compounds was based on retention times, online UV spectra, cochromatography of commercial standards (diffractaic acid and norstictic acid [Gaia Chemicals, Gaylordsville, USA]; usnic acid [Sigma, St. Louis, USA]; tenuiorin and stictic acid standards provided by Prof. Harrie Sipman [Botanical Museum, Berlin]; and peristictic, methyl norstictic, and constictic acids by Prof. John Elix [National University of Australia, Canberra]), and HPLC-MS (Agilent Technologies) (see Table 1 for HPLC-MS data). Conditions for HPLC/API-ES (positive ions) were as follows: a Hypersil RP C18 column with ID of 2 mm and 10 cm length, ES fragmentor voltage of 80, flow rate of 0.4 ml min^{-1}, and injected volume of 5 μl. The fragments of the lichen compounds produced by HPLC/API-ES are displayed in Table 1. The compounds were quantified against response curves of the above-mentioned standards. The constictic, peristictic, cryptostictic, and methyl norstictic acids were quantified by using the response curve of norstictic acid, whereas for tenuiorin derivatives, the tenuiorin standard curve was used.

Reflectance Measurements Melanic pigmentation can be quantified by measuring the reflectance (400–1,000 nm) from the lichen surface (Solhaug et al. 2003). Reflectance spectra were recorded by holding dry or wet lichen thalli against an integrating sphere (model ISP-50-REFL Ocean Optics, NL-6961 LL Eerbeek, The Netherlands) illuminated by a halogen lamp (model DH2000, OceanOptics) through a 600-μm-thick optical fiber connected to the input port of the integrating sphere. Reflectance was detected by a spectrometer (model SD2000, Ocean Optics) connected to the output port of the sphere

Table 1 HPLC-detected compounds with available HPLC-MS data for the three lichen species

Lichen Species	HPLC-MS Data
Lobaria pulmonaria	
Constictic acid	385↑, 339, 417, 827, 367, 425
Peristictic acid	411↑ (M+23), 371
Stictic acid	369↑, 401, 409, 795
Cryptostictic acid	
Norstictic acid	395 (M+23), 355↑, 449
Methyl norstictic acid	709↑, 737, 755, 795, 247
Pseudocyphellaria crocata	
Constictic acid	
Stictic acid	369↑, 401, 409, 795
Tenuiorin der 1	
Tenuiorin der 2	
Tenuiorin der 3	
Tenuiorin der 4	
Tenuiorin der 5	
Tenuiorin der 6	
Tenuiorin der 7	
Tenuiorin der 8	
Tenuiorin	165↑, 519 (M+23)
Usnea longissima	
Diffractaic acid	193↑, 374 (M+23)
Usnic acid	345↑ (M+1), 367 (M+23)

For several minor peaks there were no MS-data available/detectable.

↑=100% peak, (*M+23*)=peak identified as the molecule+a methyl group, (*M+1*)=peak identified as the molecule+a H atom.

with a 400-μm-thick fiber. Percent reflectance was calculated on the basis of a dark spectrum and a reference spectrum from a white reference tile (WS-2, OceanOptics).

Statistical Analyses The data for *L. pulmonaria* and *P. crocata*, assessed from one location only, were analyzed in a one-way ANOVA with forest type as factor and concentration of lichen compounds as effect. *Usnea longissima* was analyzed in a two-way ANOVA with site and successional stand as factors and concentration of lichen compounds as effects (SigmaStat for Windows). The reflectance data were analyzed with the nonparametric Kruskal–Wallis test (Systat).

Results

The three studied species differed substantially in their total concentration of extractable secondary compounds (Fig. 1). *Lobaria pulmonaria* had the lowest concentration with a mean value of 3.1%, *P. crocata* the highest (7.1% of dry weight). *Usnea longissima* showed an intermediate level (5.0%), but at the same time displayed the widest total range (1–11% of dry weight; Fig. 1). When recorded on area basis, however, *U. longissima*, had the highest content of secondary compounds (7.0–9.5 g m^2; Table 2), nearly three times higher than in *L. pulmonaria* with *P. crocata* in between.

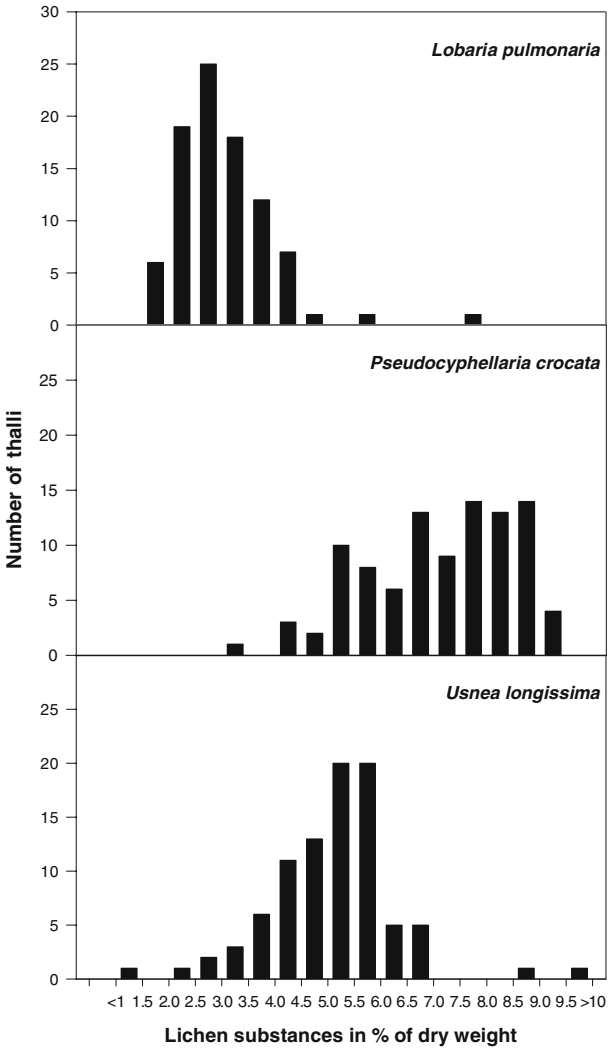


Fig. 1 Number of thalli of *L. pulmonaria*, *P. crocata*, and *U. longissima* in different concentration classes (% of DW) with respect to extractable secondary compounds

All extractable secondary compounds in *L. pulmonaria* were depsidones (Table 2). Stictic acid was the major compound, constituting 62% of the total amount of extractable secondary compounds on a dry weight basis. Constictic and peristictic acid represented together 32%, meaning that cryptostictic, norstictic, and methyl norstictic acid were minor compounds with a combined contribution of only 6% of the total secondary pool (Table 2). Only one minor compound, methyl norstictic acid, varied significantly between the successional stages, showing higher concentrations per dry weight and contents per thallus area in the clear-cuts than in forested stands.

Pseudocyphellaria crocata had a large pool of many extractable secondary compounds (Table 2). Like *L. pulmonaria*, it contained the depsidones stictic and constictic acid, but in consistently lower concentrations (7–9% of total extractable pool; Table 2). Tenuiorin, the major depside, accounted for as much as 50% of the total amount of lichen compounds in *P. crocata*. An additional eight unidentified compounds with UV and MS spectra resembling

Table 2 Concentrations (mg g^{-1}) and contents per thallus area (g m^{-2}) of all detected extractable secondary lichen substances in three old forest lichens transplanted to three different forest successional stands in an Atlantic boreal rainforest environment (Namsos)

	Concentration of Compounds (mg g^{-1} dry Weight)					Content Per Thallus Area (g m^{-2})				
	Clear-Cut	Old Forest	Young Forest	*F*	*P*	Clear-Cut	Old Forest	Young Forest	*F*	*P*
Lobaria pulmonaria										
Constictic acid	6.0± 1.0	6.9± 1.2	5.9±1.0	1.63	ns	0.71± 0.12	0.76± 0.13	0.65± 0.11	1.22	ns
Peristictic acid	3.5± 0.6	3.8± 0.7	3.5±0.6	0.05	ns	0.41± 0.07	0.43± 0.07	0.40± 0.07	0.09	ns
Stictic acid	18.6± 3.2	19.8± 3.5	17.9±3.1	0.70	ns	2.20± 0.38	2.22± 0.39	2.01± 0.35	0.52	ns
Cryptostictic acid	0.5± 0.1	0.5± 0.1	0.4±0.1	1.36	ns	0.06± 0.01	0.05± 0.01	0.05± 0.01	2.44	ns
Norstictic acid	0.9± 0.2	0.9± 0.2	0.8±0.1	1.18	ns	0.11± 0.02	0.10± 0.02	0.09± 0.02	1.98	ns
Methyl norstictic acid	0.6± 0.1	0.5± 0.1	0.5±0.1	7.52	0.001	0.08± 0.01	0.06± 0.01	0.05± 0.01	7.56	0.001
Total	30.2± 5.3	32.4± 5.6	29.0±5.0	1.16	ns	3.55± 0.62	3.62± 0.63	3.26± 0.57	0.90	ns
Pseudocyphellaria crocata										
Constictic acid	4.2± 0.3	2.7± 0.3	3.6±0.4	5.39	0.006	0.38± 0.03	0.25± 0.00	0.31± 0.04	4.21	0.019
Stictic acid	2.4± 0.2	1.7± 0.2	2.1±0.2	4.79	0.010	0.21± 0.01	0.16± 0.00	0.18± 0.02	3.20	0.045
tenuiorin der 1	2.5± 0.1	2.8± 0.1	3.0±0.1	4.86	0.010	0.21± 0.01	0.27± 0.00	0.27± 0.01	8.66	<0.001
tenuiorin der 2	0.4± 0.0	0.3± 0.0	0.4±0.0	1.46	ns	0.03± 0.00	0.03± 0.00	0.04± 0.00	0.54	ns
tenuiorin der 3	1.1± 0.1	1.2± 0.1	1.2±0.1	1.66	ns	0.09± 0.01	0.12± 0.00	0.11± 0.00	4.03	0.021
tenuiorin der 4	7.5± 0.5	10.7± 1.0	10.5±0.8	5.14	0.008	1.07± 0.06	1.04± 0.01	0.94± 0.08	7.71	<0.001
tenuiorin der 5	11.9± 0.6	9.3± 0.5	10.9±0.8	4.47	0.014	1.07± 0.06	0.91± 0.01	0.97± 0.08	1.87	ns
tenuiorin der 6	0.6± 0.1	0.8± 0.1	0.9±0.1	3.10	0.050	0.05± 0.00	0.08± 0.00	0.08± 0.01	3.40	0.037
tenuiorin der 7	2.7± 0.1	2.4± 0.2	2.4±0.1	1.37	ns	0.24± 0.01	0.24± 0.00	0.22± 0.02	0.60	ns
tenuiorin der 8	3.1± 0.2	2.3± 0.2	2.5±0.2	5.66	0.005	0.28± 0.02	0.23± 0.00	0.23± 0.02	2.62	ns
Tenuiorin	39.0± 2.1	33.4± 1.4	33.4±2.0	3.27	0.042	3.53± 0.21	3.34± 0.03	3.04± 0.21	2.39	ns
Total	75.4± 2.6	67.5± 1.9	70.9±2.7	3.09	ns	5.70± 0.25	5.77± 0.04	5.41± 0.26	0.62	ns
Usnea longissima										
Diffractaic acid	5.8± 0.4	6.2± 0.3	6.2±0.3	0.51	ns	0.94± 0.06	1.13± 0.07	1.00± 0.05	2.48	ns
Usnic acid	49.4± 3.1	43.9± 2.4	38.3±2.3	6.82	0.002	8.16± 0.40	8.31± 0.57	6.04± 0.41	7.38	0.001

Table 2 (continued)

	Concentration of Compounds (mg g^{-1} dry Weight)					Content Per Thallus Area (g m^{-2})				
	Clear-Cut	Old Forest	Young Forest	*F*	*P*	Clear-Cut	Old Forest	Young Forest	*F*	*P*
Total	55.1± 3.4	49.8± 2.6	44.3±2.4	5.80	0.004	9.10± 0.43	9.45± 0.62	7.03± 0.42	6.93	0.002

those of tenuiorin were found, representing more than 40% of the total pool. These are referred to as tenuiorin derivatives 1 to 8. The yellow pulvinic acid derivate calycin, present only in cortex-deficient soredia and small pseudocyphellae (unpublished data), was not detectable in extracts of whole thalli. As many as 8 out of the 11 compounds in *P. crocata* differed significantly between forest stands (Table 2) when calculated on a dry weight basis, whereas 6 compounds were affected on area basis. The trend, however, varied among the compounds. The content of constictic acid, stictic acid, tenuiorin derivatives 5 and 8, and tenuiorin (the three latter only per dry weight) was highest in the clear-cut transplants, whereas tenuiorin derivatives 1, 3 (only per area), 4, and 6 were lowest on the clear-cuts (Table 2).

The melanic pigmentation in the upper cortex of both *L. pulmonaria* and *P. crocata* was consistently darker, evidenced as lower reflectance, in clear-cut transplants than in those from the old and the young forest (Fig. 2). This was the case for both visible and far-red wavelength ranges ($P<0.001$, ANOVA; data not shown), and for both air-dry and hydrated thalli ($P<0.001$, ANOVA; data not shown).

Usnea longissima contained two detectable secondary lichen compounds, the medullary depside diffractaic acid and the cortical dibenzofuran derivative usnic acid (Table 2). Usnic

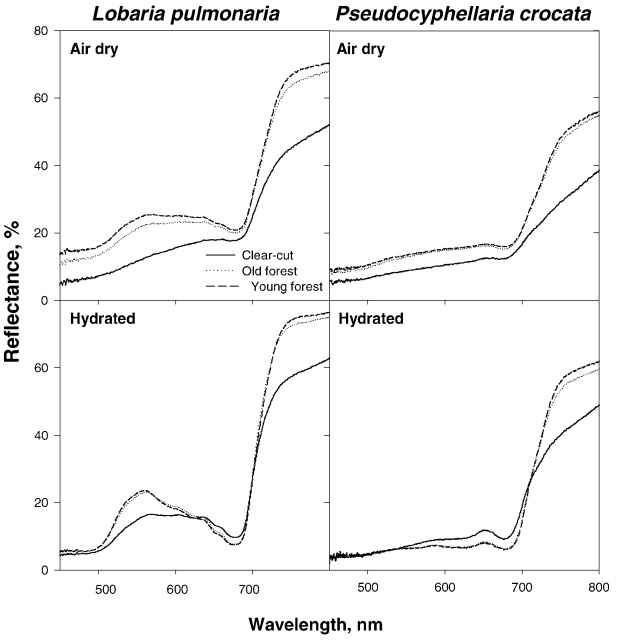


Fig. 2 Reflectance (%) from the upper surface of air dry and hydrated *L. pulmonaria* and *P. crocata* thalli transplanted to clear-cuts, old forests, and young forests in an Atlantic rainforest environment in W Norway

acid was the major compound, constituting more than 90% of the total amount of secondary substances. Because the transplantation of *U. longissima* was repeated at three locations along a regional climatic gradient from rain forests to drier continental forests, a two-way ANOVA that also included the rainfall gradient was run. The successional stage had a strong influence on the secondary chemistry, as the concentration of usnic acid was highest in thalli from the clear-cuts compared with both old and young forests and the concentration of diffractaic acid was slightly lower in clear-cut transplants (Fig. 3). The steep rainfall gradient (locality) did not give any significant contribution alone (Fig. 3). However, there was a significant interaction effect because the old forest transplants in the Atlantic location had elevated content of usnic acid per thallus area compared to the clear-cut as well as young forest transplants. No such interactions were seen for the diffractaic acid (Fig. 3).

There was no significant correlation between the concentration of extractable lichen substances and the dry weight gain of lichen thalli for any of the investigated species (data not shown).

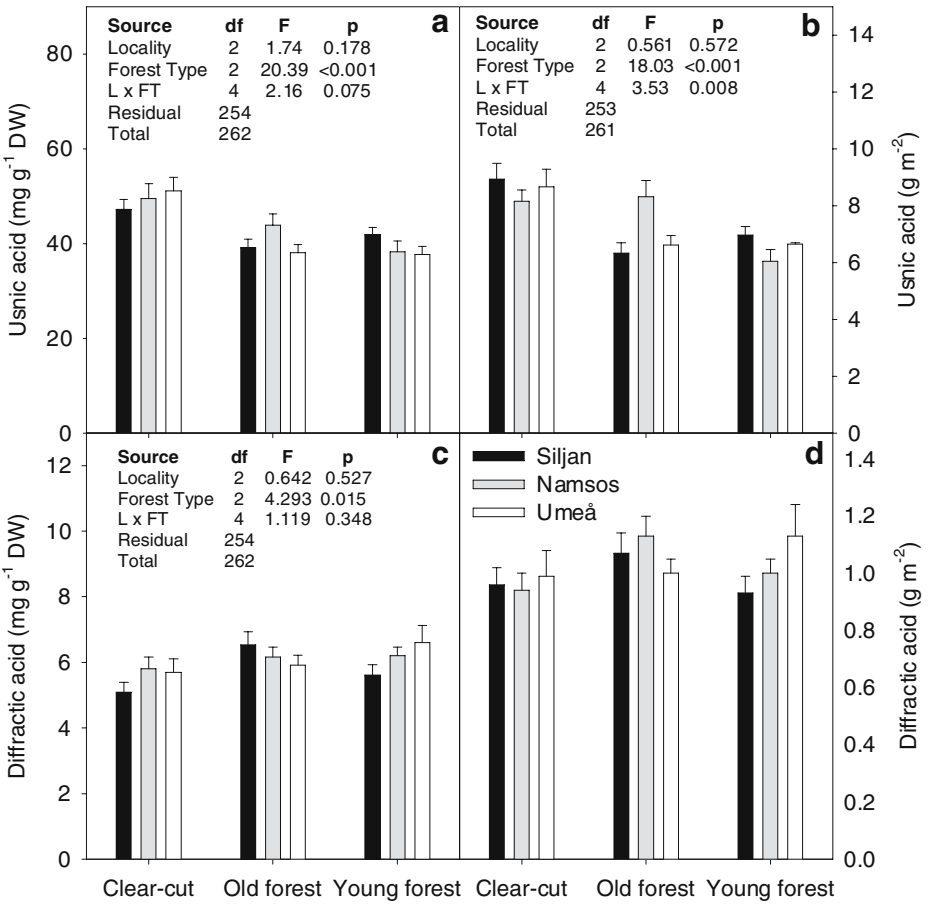


Fig. 3 Secondary compounds in *U. longissima* transplants from three successional stands (clear-cut, old, and young forest) and three climatic zones [Suboceanic (Siljan), Atlantic (Namsos), and Continental (Umeå) zones]. Means±SE are given as concentrations (**a**, **c**) and content per area (**b**, **d**). **a**, **b** Usnic acid. **c**, **d** Diffractaic acid. A two-way ANOVA table is given when at least one factor gives a significant effect

Discussion

The cortical lichen substances, usnic acid in *U. longissima*, and the melanic pigmentation in *L. pulmonaria* and *P. crocata* increased with increasing canopy openness, which suggests common functional roles of the dibenzofurane derivative and melanin as light protection. Browning of light-exposed upper cortices is a well-known phenomenon for some tripartite (*L. pulmonaria*; Solhaug et al. 2003) and green-algal lichens (Nybakken et al. 2000; Nybakken and Julkunen-Tiitto 2006), but has not previously been quantified for purely cyanobacterial lichens like *P. crocata*. Melanins are located in an intact, upper cortex screen that underlies photobionts, thus potentially protecting against UV as well as visible radiation (Gauslaa and Solhaug 2001). Usnic acid in solution strongly absorbs UV-B, some UV-A, and small amounts of blue light (McEvoy et al. 2006). However, extractable cortical compounds like usnic acid are located as numerous tiny crystals outside fungal hyphae. Such external crystals cause extensive scattering of light, meaning that crystals of usnic acid probably screen a wider range of visible light than recorded when they are dissolved. Measurements of the transmittance in a thin crust of usnic acid crystals, spread out on a UV-transmitting Plexiglas, show that the crystals significantly screen visible wavelength ranges at wavelengths where no absorption occurs in solution (K.A. Solhaug, unpublished data). *Usnea* species are too finely constructed to allow preparation of cortex fragments for measuring transmittance, but measurements of transmittance through upper cortices of the usnic acid containing *Nephroma arcticum* (Maria McEvoy, unpublished data) showed up to 50% screening in the visible range. *Usnea longissima* did not experience any photoinhibitory damage during the transplantation on exposed clear-cuts (Gauslaa et al. in press), despite the fact that *Usnea* species are inhabited by trebouxioid photobionts, which are considered to be highly susceptible to high light (Ahmadjian 1959). The *U. longissima* transplants had the highest growth rates in clear-cuts (dry weight gain: 13, 26, and 38% in young forest, old forest, and clear cut, respectively; Gauslaa et al. in press), where the content of usnic acid is highest (Table 2). By contrast, the two foliose species grew best in the old forest (dry weight gain: *L. pulmonaria* 29, 35, and 26%; *P. crocata* 32, 36, and 23% in young forest, old forest, and clear cut, respectively) and showed strong chlorophyll degradation in the clear-cuts (Gauslaa et al. in press), despite the rapid and strong formation of cortical melanins in the thalli. These contrasting responses suggest that a solar screen formed by tiny bright and reflecting crystals of usnic acid is a safer solar radiation screen compared with a melanic screen, likely because dark melanic pigments may jeopardize photochemical processes with excess heat because of their high absorbance of solar radiation (Gauslaa and Solhaug 1999, 2001).

Many medullary compounds are located mainly in the photobiont layer. Their position has been claimed to be consistent with screening of solar radiation (Fahselt and Alstrup 1997). Our overall negative results (Table 2) provide evidence against a general light-protecting function for these UV-B-absorbing medullary compounds. However, many individual medullary compounds did show stand-specific responses, particularly in *P. crocata*, although these trends were generally weak or with contrasting directions (Table 2). This might result from changed priorities in metabolism because of different environmental conditions experienced by lichens in the studied forest successional stages. Stictic acid has a growth-retarding effect on larvae of the polyphagous insect herbivore *Spodoptera littoralis* (Emmerich et al. 1993; Giez et al. 1994). Other than this, there is little knowledge of any functional role of the individual compounds found in *L. pulmonaria* and *P. crocata*. Because many compounds are unidentified, and synthetic pathways are not clear, we refrain from postulating about these responses in further detail.

In *U. longissima*, the medullary depside diffractaic acid responded in an inverse pattern to the cortical dibenzofuran derivative usnic acid (Fig. 3). In clear-cuts, where much carbon is spent for new growth as well as for synthesis of usnic acid, the production of diffractaic acid probably has a lower priority. However, various depsides have been shown to have toxic or deterrent effects on herbivores (Rundel 1978; Giez et al. 1994). Increased concentration of diffractaic acid in the shade could be a response to increased pressure from lichen-feeding invertebrates and decomposing microorganisms under shading canopies. The opposite trends, thus, suggest different roles for cortical and medullary substances in *U. longissima*.

There was no difference in concentration of lichen substances in *U. longissima* along the rainfall gradient (Atlantic–suboceanic–continental locations). Quantification of canopy cover based on hemispherical digital photos (Gauslaa et al. in press) showed that differences among the localities in indirect solar radiation were much lower than the differences among successional stages. This suggests that the concentration of secondary compounds in *U. longissima* is more dependent on canopy openness than rainfall. We would expect humidity (time in the wet and active state; Palmqvist 2000) and amount of sunlight to be important for the build up of carbon available for production and time for synthesis of the secondary compounds. However, we have no data on the actual amount of solar irradiation for the wet, physiologically active periods of the thalli. Open sites, relative to forested stands, are likely to experience frequent cooling below the dew point during nights, meaning that light availability and humidity may be confounding factors.

In summary, the synthesis of the UV-B absorbing cortical compounds usnic acid and melanins seems to be part of an acclimation to increased light exposure. However, usnic acid seems to be a more flexible screen than the brown melanins because it allows maximal thallus growth in fully exposed sites. The medullary compounds in the studied species seem hardly to function as solar screens despite their strong absorbance in the UV-B range.

Acknowledgements We thank Kristin Palmqvist, Håkon Holien, Lise Cats Myhre, Olga Hilmo, Mikael Ohlson, and Camilla Iversen for help with collection of lichen samples, set-up and/or maintenance of the experiments. We are also indebted to Professor Harrie Sipman at the Botanical Museum in Berlin, Germany, for giving us standards of the lichen compounds tenuiorin and stictic acid; and to Professor John Elix, University of Canberra, Australia and Professor Riitta Julkunen-Tiitto, University of Joensuu, Finland for help with identification of lichen substances. This study was supported by the Norwegian Research Council (project number 15778/V40).

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of CA and AT may be due to the reactive hydroxyl groups at positions 2 and 4 of ring A, instead of the less reactive methoxyl at the same positions in both BO and MA. In the field comparisons, total quantified phenolics were significantly higher near Mexico City for both lichens, except for the tolerant *R. asahinae* at one site. Nevertheless, only the latter species had significantly increased PAC values at all sites near the city. This result is explained by species-dependent changes in individual phenolics. At the polluted sites, *R. asahinae* had consistently higher contents of its most reactive phenolic, US, with values approximately twice that of the control site. In contrast, *P. stuppeum* only increased its less reactive SA (26–35%), but this was counteracted by CA and, to a lesser extent, AT degradation. Thus, the substantial increase in US at the polluted sites appears to be associated with the current ecological success of *R. asahinae* near the city. On the other hand, the inability of *P. stuppeum* to overcome degradation of its most reactive phenolic (CA) at the same sites seems to partially explain the declining status of this lichen. These results provide evidence for a protective mechanism in lichens against air pollution based on secondary metabolites, which may eventually determine which species survive in forests stressed by oxidative air pollution.

Keywords Lichen secondary metabolites · Ozone reactivity · Antioxidant activity · Free radical scavenging activity against DPPH$^{\bullet}$

Introduction

Lichens are an ancient, large, and ubiquitous group of symbiotic fungi that obtain carbon from specific photosynthetic partners, which are either green algae or cyanobacteria, or both in some cases (Nash III 1996). Part of that carbon is used to synthesize a variety of secondary metabolites, also known as lichen substances. There are about 850 identified lichen secondary metabolites, many of which are phenolics deposited as crystals on the fungal cell walls (Huneck and Yoshimura 1996). Depending upon the species, secondary metabolites constitute 0.1–25% of the lichen thallus on a dry weight basis, but values of 5–10% are more frequent (Dayan and Romagni 2001). Each lichen species has a defined set of secondary metabolites, which can vary quantitatively and qualitatively as influenced by environmental factors such as light intensity and moisture and by intrinsic factors such as genetics, age, and vitality (BeGora and Fashelt 2000). The variability of secondary metabolites induced by the environment demonstrates the importance of these substances for the ecological adaptiveness of lichens.

Some evidence for the biological activity of the lichen substances derives from studies looking for natural compounds with antibiotic, antitumoral, or antioxidant activity for pharmaceutical and food uses (e.g., Hidalgo et al. 1994; Lauterwein et al. 1995; Ingólfsdóttir et al. 1998; Müller 2001; Gülçin et al. 2002; Toledo et al. 2003). Lichen compounds are also being tested as alternative, environmentally friendly, pesticides for agriculture (Dayan and Romagni 2001). Most functions attributed to these metabolites are protective, including filtration of harmful ultraviolet (UV) radiation, deterrence of herbivores, defense against microorganisms, and as allelopathic compounds (e.g., Clark et al. 1999; BeGora and Fashelt 2000; Toledo et al. 2003).

It has been suggested that lichen secondary metabolites may respond to environmental pollution (e.g., Silberstein et al. 1996; Caviglia et al. 2001; MacGillivray and Helleur 2001;

Cabral 2002). In the case of air pollution, lichens are used frequently as biomonitors or bioindicators but their phenolics are rarely measured as response variables. The potential of phenolics for studying lichen responses to air pollution can be inferred from their antioxidant properties and their ability to form insoluble complexes with some metals (Silberstein et al. 1996; Takani et al. 2002). The protective role of phenolics against reactive oxygen species (ROS), including O_3, is well documented for vascular plants (Ranieri et al. 1996). It is, however, questioned whether lichen phenolics play a similar role basically because of their extracellular location (e.g., Kranner et al. 2005). Nevertheless, because of this location, we hypothesized that lichen phenolics may provide some degree of protection to vital lichen cell compartments by reducing the amount of environmental oxidants at the cell wall surface. A possible mechanism for that to occur could be provided by surface gas–solid oxidation reactions. These reactions are rather common to both inert and biological surfaces, especially when organic compounds containing such reactive groups as double-bonds, aromatic hydroxyls, halogens, or carbonyls are present. An example of such surface reactions is the ozonolysis of polyaromatic hydrocarbons on aerosol particles (Pöschl et al. 2001; Hearns and Smith 2004).

Although deleterious effects of O_3 on vascular plants are well-documented (e.g., Krupa et al. 2001), in the case of lichens, such evidence is scarce and contradictory (e.g., Nash III and Sigal 1979; Ross and Nash III 1983; McCune 1988; Scheidegger and Schroeter 1995; Tarhanen et al. 1997). There are pollution-sensitive and pollution-tolerant lichen species (Silberstein et al. 1996), but insufficient consideration has so far been given to the mechanisms that protect resistant lichens from the effect of toxic exposure. A field study conducted in Canada by MacGillivray and Helleur (2001) looked for correlations between lichen phenolic contents and ground-level concentrations of O_3 (17–31 ppb). They found that phenolic contents tended to decrease with O_3 concentration, except for salazinic acid (SA), which increased slightly in *Usnea dasypoga*. This latter increase was interpreted by the authors as a protective biochemical response, and they proposed that SA could be a potential biomarker for O_3 pollution. Such an interpretation is a complex hypothesis requiring more field and laboratory evidence. If it were statistically accepted and extended to other phenolics, it could eventually be possible to take advantage of the great diversity of lichen secondary metabolites to identify responses toward specific air pollutants, an unsolved problem in most lichen field studies.

We report here experimental and observational results from a study that addressed two basic questions related to that hypothesis: (a) Do lichen phenolics react with O_3 and exhibit free radical scavenging properties? and (b) Do lichen populations chronically exposed to heavy oxidative air pollution differ significantly in phenolic profiles from unexposed populations? The first question was answered by kinetic experiments to determine the reactivity between six different lichen phenolics and ozone and their free radical scavenging power. Three major phenolics of two epiphytic lichen species with contrasting tolerance to heavy oxidative air pollution from Mexico City were studied. For this purpose, solutions of pure isolates of these compounds were exposed in vitro to O_3 and diphenyl picryl hidrazyl ($DPPH^{\bullet}$). This latter compound is commonly used to assess the free radical scavenging capacity of whole plant or lichen extracts, as well as specific plant phenolics (e.g., Brand-Williams et al. 1995; Arlorio et al. 2005; Behera et al. 2005). The second question was addressed by comparing the field contents of the same phenolics in samples of the same lichen species between fir forests chronically exposed to urban emissions from Mexico City and a relatively unpolluted and well-conserved fir forest. One more site comparison was based on the potential antioxidative capacity (PAC) provided to the lichens by the measured phenolics.

Methods and Materials

Study Area and Lichen Material The Mexico City Metropolitan Area (MCMA) is the largest, most populated (ca. 20 million people) and most air-polluted urban area in Mexico. During the last 15 years of systematic air monitoring, O_3 has been the most critical pollutant in this area, and this situation was probably similar in previous years. That is attributed to elevated local emissions of O_3 precursors (e.g., VOCs and NO_x) by over 3.5 million vehicles and a variety of industrial and domestic emission sources powered by fossil fuels. Ozone photochemical formation is enhanced by the high solar irradiation received at this high-elevation tropical city and topographical and meteorological conditions that restrict natural ventilation (Blake and Rowland 1995; Bravo and Torres 2002; GDF 2002). The MCMA (2,240 m above sea level) is located at the SW corner of the Mexico Basin, which is surrounded almost entirely by forested, high-elevation mountains (2,000 m above the urban floor, Fig. 1). This region provides a good opportunity to study effects from urban emissions on the natural vegetation. The oak and conifer forests located to the S–SW of the MCMA are the most affected by air pollution because they are closer to the urban area and lie predominantly downwind of it. The current knowledge on this topic was recently compiled by Fenn et al. (2002). Ozone monitoring and studies on oxidative injury to sacred fir (*Abies religiosa*) at Desierto de los Leones, where 100 ppb O_3 maximum hourly concentrations occur throughout most of the year along with 200–300 ppb monthly peaks (Alvarado-Rosales and Hernández-Tejeda 2002), indicate that oxidant air pollution is a major local forest disturbance factor.

The lichens *Ramalina asahinae* and *Parmotrema stuppeum* (Fig. 2) were chosen because they represent, according to data of Zambrano and Nash III (2000), two contrasting cases of

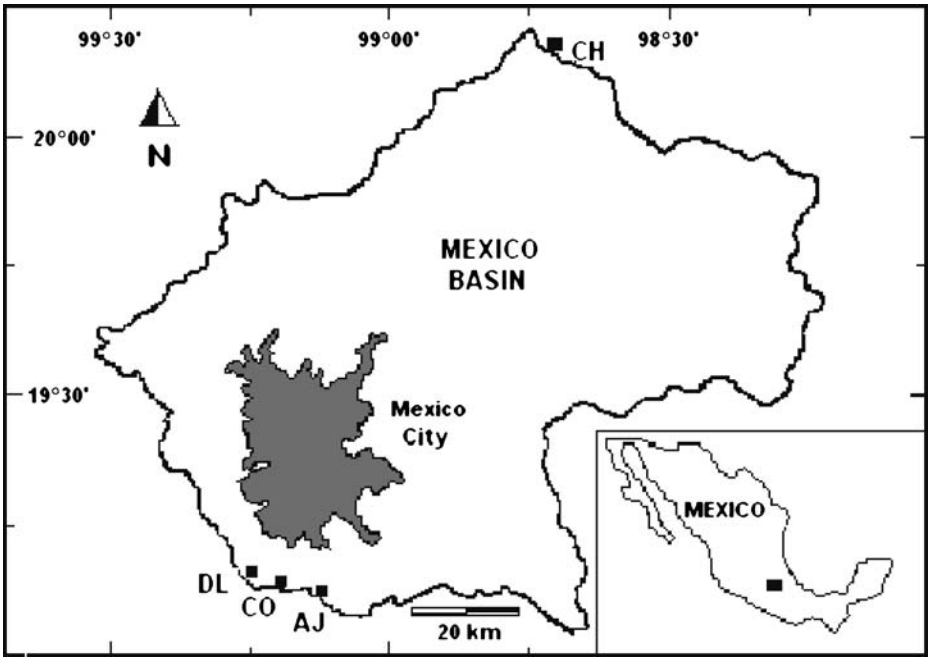


Fig. 1 Study sites. Ajusco Volcano, *AJ*; Dinamos de Contreras, *CO*; El Chico National Park, *CH* (control site); Desierto de los Leones, *DL*

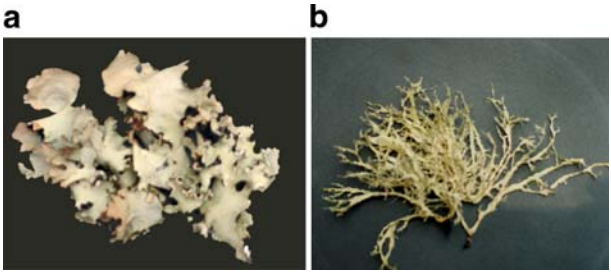


Fig. 2 Photos of lichens **a** *Parmotrema stuppeum* and **b** *Ramalina asahinae*

species tolerance toward chronic exposition to high levels of air pollution in forests near Mexico City. *Ramalina asahinae*, cited as *R*. cf. *farinacea* by Zambrano and Nash III (2000), is the dominant and thus tolerant, epiphytic lichen of sacred fir. In contrast, and compared to similar sites less exposed to the urban emissions, *P. stuppeum* is severely depleted in abundance. However, this species is abundant in fir forests far from the reach of urban emissions. For example, at El Chico National Park (CH, Fig. 1), where the sacred fir forests still sustain healthy epiphytic lichen communities, *P. stuppeum* is the dominant species; whereas, *R. asahinae* ranks fourth in abundance (Zambrano and Nash III 2000).

All lichen samples for field comparisons of phenolic contents were collected between mid-January and mid-February, 2004. After a nested experimental design, three fir forests located 8–12 km to the S–SW of Mexico City were compared to a control site ca. 100 km N of the city (Fig. 1, Table 1). Lichens were sampled only from the trunks of sacred firs to minimize variability because of substrate differences. Within-site variation was assessed by sampling three forest stands per site. Stands were separated by at least 150 m. A lichen sample was defined as the material of each species (about 200 mg air-dry weight) obtained from one tree. Nine samples per stand were obtained; i.e., 27 samples per site. *P. stuppeum* was not sampled at one site near Mexico City (Ajusco Volcano), where this species is almost extinct. Air-dried lichen samples were carried to the laboratory in paper bags. Taxonomical identification was done by conventional lichenological techniques.

Preparation of Lichen Extracts for Field Comparisons Samples (ca. 30 mg) from the newest, marginal thalli portions were desiccated on drierite for 48 h. Dry, mortar-ground samples (20 mg) were extracted for 1 h with 5 ml of 1:1 acetone and methanol (Burdick & Jackson) and spiked with benzophenone (BZ; 0.25 mg ml^{-1}, Sigma Aldrich) as internal standard. Extracts were filtered through a millipore™ membrane filter (0.45 μm), and the filtrates were submitted to high-performance liquid chromatography (HPLC). A chromatogram of a *P. stuppeum* extract is shown in Fig. 3.

Table 1 Location of study sites

Site	Coordinates	Distance (km), Orientation[a]	Elevation (m.a.s.l.)
CH	20°10′N; 98°43′W	100 NNE	3,000–3,050
DL	19°19′N; 99°20′W	8 SW	3,200–3,350
AJ	19°14N; 99°15′W	12 SW	3,250–3,400
CO	19°15′N; 99°16′W	10 SW	3,200–3,300

CH El Chico National Park (control); *DL* Desierto de los Leones Nacional Park; *AJ* Ajusco Volcano; *CO* Dinamos de Contreras

[a] From Mexico City

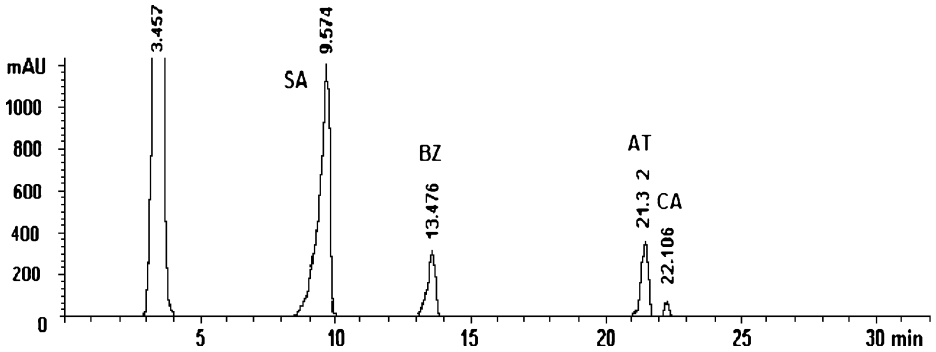


Fig. 3 HPLC chromatogram for *P. stuppeum* extract. *SA*, salazinic acid; *BZ*, benzophenone; *AT*, atranorin, and *CA*, chloroatranorin

Isolation and Characterization of Lichen Phenolics Six lichen phenolics (LP) were tested for antioxidant capacity. All were isolated by conventional chromatographic techniques (Ramaut et al. 1978; Jayaprakasha et al. 1998) from materials collected at the control site. SA, atranorin (AT), and chloroatranorin (CA) were isolated from *P. stuppeum*; boninic acid (BO), 2-*O*-methylsekikaic acid (MA), and usnic acid (US) were obtained from *R. asahinae* (Fig. 4). The purity of isolates was confirmed by HPLC, and their identity was determined

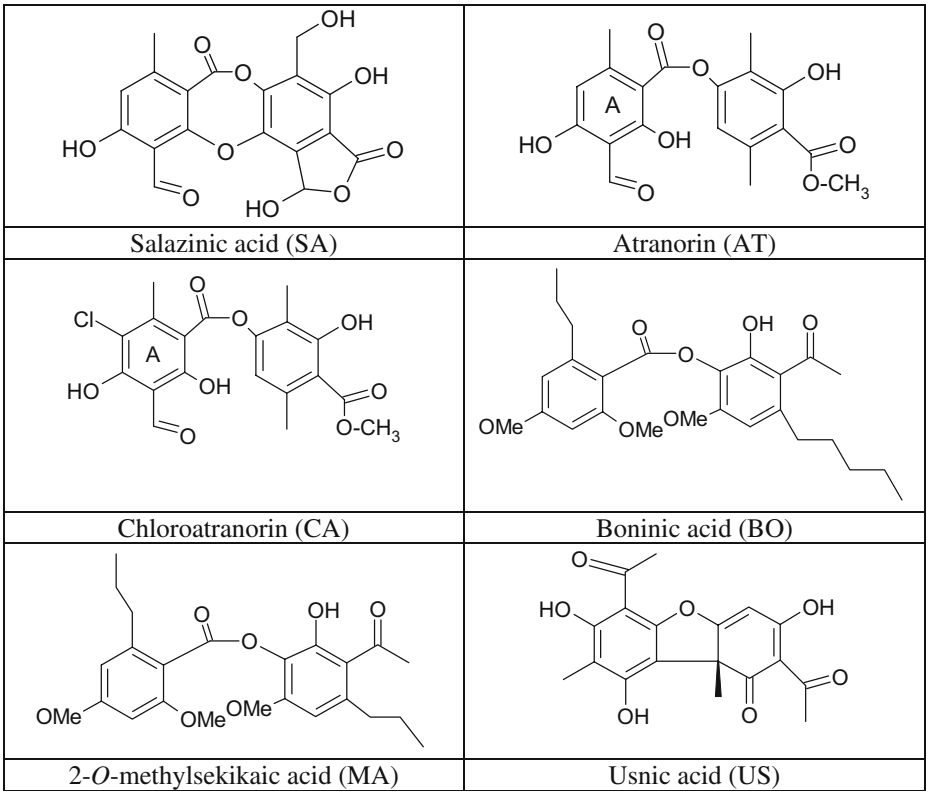


Fig. 4 Lichen phenolics included in this study

by comparing measured UV, infrared, nuclear magnetic resonance, and mass spectrometry values with those reported by Eifler-Lima et al. (2000) and Huneck and Yoshimura (1996).

HPLC Analysis of LP Quantification of LP for field comparisons and ozonation experiments was conducted with a liquid chromatograph (Agilent HP, 1100 series) equipped with diode-array detector and Nucleosil 100-5 C18 column (Agilent, 250×4.0 mm, 5 μm particle, 100 Å pore). Runs started with 50% methanol (Burdick & Jackson) and 50% water (Fermont) added with 1% orthophosphoric acid (Baker). The flow rate was 0.7 ml min^{-1}. Methanol was increased to 70% within 7 min and to 100% during the after 13 min. Runs continued isocratically for six additional minutes. Finally, methanol was brought to 50% within 1 min and the column flushed for 5 min. Injections were administered in 20 μl volumes, and the detector wavelength was set at 254 nm. An internal standard, benzophenone, was included in each sample.

Kinetic Studies of LP with Ozone Reaction rates of lichen phenolics with O_3 were empirically determined by aqueous ozonation at 20°C, under conditions where the initial concentration of lichen phenolic was low compared to the initial ozone concentration: $[LP]_0 << [O_3]_0$. Molar aqueous solutions of each LP (SA, 503.1×10^{-6}; AT, 105.5×10^{-6}; CA, $1{,}128\times10^{-6}$; BO, $1{,}305.0\times10^{-6}$; MA, 364.0×10^{-6}, and US, $1{,}074.0\times10^{-6}$) added with benzophenone as internal standard were mixed with ozonated water in glass reaction vessels (350 ml). Stock ozonated water solutions were prepared by bubbling water with O_3 from a Polyozone generator (Polimetrics, Inc. Model T-816, 330 series) fed with pure commercial oxygen. The initial concentration of O_3 in water was determined by the iodine method (Skoog and West 1990). Because $[O_3]$ decreased by less than 5% during experiments, as determined by final measurements, it can be assumed that $[O_3]$ was constant during the experiments.

After mixing solutions, a subsample was drawn at different times to quantify the remaining [LP]. To minimize further ozonolysis of LP before the HPLC measurements, the residual O_3 was eliminated by bubbling the sample with pure oxygen. All experiments included procedural blank and unozonated sample solutions.

Under the used reaction conditions (i.e., $[O_3]$ in large excess compared to [LP]), the less concentrated reactant decays exponentially, and thus a pseudo-first-order kinetics can be assumed. This is a common assumption in ozonation studies (e.g., McDowell et al. 2005). According to Eq. 1

$$[\mathrm{LP}] = [\mathrm{LP}]_0 e^{-k_{\mathrm{obs}}t} \tag{1}$$

where [LP] is the lichen phenolic concentration at any time (t), as quantified by HPLC, $[LP]_0$ is the lichen phenolic concentration at time zero, and k_{obs} is the pseudo-first-order rate constant.

The pseudo-first-order rate constant was calculated from Eq 2, where ln[LP] is linearly dependent on time in all cases and from the slope of this plot.

$$\ln[\mathrm{LP}] = \ln[\mathrm{LP}]_0 - k_{\mathrm{obs}}\, t \tag{2}$$

The second-order-rate constant (k_2) was calculated for each LP from Eq. 3

$$-\mathrm{d}[\mathrm{LP}]/\mathrm{d}t = k_{\mathrm{obs}}[\mathrm{LP}] = k_2[\mathrm{O_3}][\mathrm{LP}] \tag{3}$$

Free Radical Scavenging Activity of LP by the DPPH• Method The free radical scavenging activity of lichen phenolics was determined by reaction with the DPPH• free radical at

20°C, according to Brand-Williams et al. (1995). Briefly, fresh DPPH$^{\bullet}$ methanol solutions were mixed with fresh methanol solutions of each LP. The amount of reacted DPPH$^{\bullet}$ was calculated from the decrease in absorbance at 515 nm as measured with an UV-visible spectrophotometer (Perkin Elmer, Lambda 25) and using a previously determined calibration curve. Absorbance was recorded at 0.1 min after mixing the reactants. Afterwards, it was recorded every 15 min until reaching the reaction plateau. Each LP required a different concentration interval to estimate its reaction rate. These intervals were determined by preliminary experiments (in moles of LP/mole DPPH$^{\bullet}$): SA, 5.0–180.0; AT, 0.25–80.0; BO, 5.0–180.0; MA, 1.0–80.0; and US, 0.25–80.0. Ascorbic acid (AA) was used as positive control at a concentration range of 0.069–0.509. The antiradical power (ARP) of each LP was calculated following Brand-Williams et al. (1995).

Kinetic Studies of LP with DPPH$^{\bullet}$ Plots and fittings of experimental data were done with the Table Curve 2D v5.01 program following Suja et al. (2004). Experiments were conducted at 20.0°C. The second-order rate constants were calculated for each compound as a measure of radical scavenging capacity. To determine constants, each phenolic required a different concentration interval with respect to DPPH$^{\bullet}$ (number of moles of LP/mole DPPH$^{\bullet}$): 19.1–94.2 for SA; 19.7–82.7 for AT; 22.1–33.1 for CA; 42.64–97.53 for BO; 21.4–87.7 for MA; and 20.2–51.0 for US. The decay of [DPPH$^{\bullet}$] was assumed to follow a pseudo-first-order kinetics under the condition that $[DPPH^{\bullet}]_0 \ll [LP]_0$. According to Eq. 4

$$[DPPH^{\bullet}] = [DPPH^{\bullet}]_0 e^{-K_{obs}t} \quad (4)$$

k_{obs} and second-order rate constants (k_2) were calculated as for the ozonolysis of lichen phenolics.

Results and Discussion

The epiphytic lichens *R. asahinae* and *P. stuppeum* were studied to explore whether their major secondary metabolites are involved in the tolerance of the first species and the sensitivity of the second species to chronic habitat exposure to high levels of oxidative air pollution from Mexico City.

Antioxidant and Free Radical Scavenging Activity of LP The six tested phenolics had some degree of in vitro antioxidant activity and free-radical scavenging capacity. Table 2 shows the experimental second order reaction rate constants (k_2-O_3 and k_2-$_{DPPH^{\bullet}}$) and the ARP of these lichen compounds. ARP was expressed as $1/EC_{50}$ (Brand-Williams et al. 1995), where EC_{50} is the phenolic concentration that decreases the DPPH$^{\bullet}$ concentration by 50% as illustrated in Fig. 5. According to the kinetic constants (Table 2), the following decreasing reactivity trends were determined for the studied phenolics: CA>>AT>US>SA≥BO≥MA for O_3 and CA> AT>US>MA>SA=BO both for the DPPH$^{\bullet}$ and the ARP criteria. The reactivity ranking for the three most reactive compounds was thus concordant between k_2-$_{O3}$ and k_2-$_{DPPH^{\bullet}}$, but minor ordering discrepancies occurred for the less reactive ones, which was probably because of reaction details that merit further analysis. The most reactive secondary metabolite of *P. stuppeum*, CA, disappeared so rapidly upon contact with O_3 that k_2-O_3 could not be determined under our experimental conditions. However, the DPPH$^{\bullet}$ experimental model showed that CA scavenged this free radical at rates 150 and 733% higher than AT and SA, respectively. Atranorin reacted with O_3 and DPPH$^{\bullet}$ at about twice the rate that SA reacted

Table 2 Second-order rate constants (M^{-1} s^{-1}) for the reaction of lichen phenolic compounds with ozone (k_2-O_3) or DPPH$^{\bullet}$ (k_2-DPPH$^{\bullet}$)

Lichen	Compound	k_2-O_3±sd[a]	k_2-DPPH$^{\bullet}$±sd[b]	ARP[c]
R. asahinae	BO	50±2	0.0048±0.0004	0.0144
	MA	46±3	0.010±0.001	0.0519
	US	79±2	0.014±0.001	0.1124
P. stuppeum	SA	53±3	0.0060±0.0004	0.0144
	AT	102±3	0.020±0.002	0.5761
	CA	>>>102	0.050±0.001	0.5887

[a] *sd* Standard deviation, $n=3$

[b] $n=5$

[c] Antiradical power

with the same stressors. In the case of *R. asahinae*, the most reactive compound was US. It reacted with O_3 at rates 58 and 72% higher than BO and MA, respectively, and it reacted with DPPH$^{\bullet}$ at rates 192 and 40% higher than BO and MA.

It is not yet possible to compare directly these reactivity rates with results from other lichen phenolic studies because of different experimental models among them (e.g., Hidalgo et al. 1994; Jayaprakasha and Rao 2000; Toledo et al. 2003). Some authors tested whole lichen extracts with DPPH$^{\bullet}$ (e.g., Gülçin et al. 2002; Odabasoglu et al. 2004), in which case, the antioxidant capacity of individual phenolics was not determined. With whole lichen extracts, the antioxidant capacity of phenolics is confounded with that from other cell compounds in the mixtures. Lichen depsidones have been reported as more efficient antioxidants (Hidalgo et al. 1994) and also as more cytotoxic (Correche et al. 2004) than depsides. Our results show that depsides (AT and CA) had higher antioxidant capacity than SA (a depsidone) and the meta-depsides BO and MA. In view of the limited experimental and

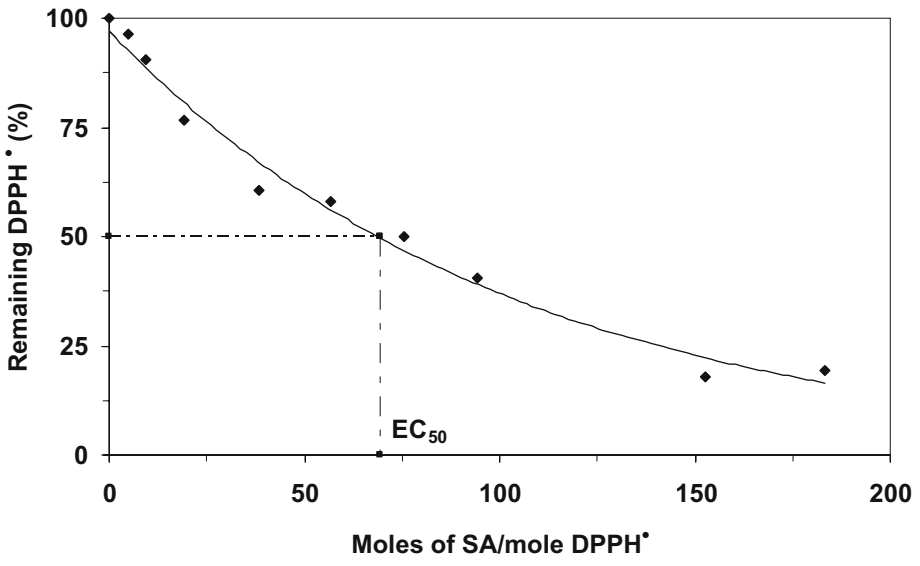


Fig. 5 Disappearance of DPPH$^{\bullet}$ as a function of salazinic acid (*SA*) concentration. EC_{50} was the SA concentration causing a 50% decrease in DPPH$^{\bullet}$ absorbance

theoretical data available on the antioxidative properties of lichen phenolics, it is still not possible to generalize at the chemical group level. However, it can be hypothesized that AT and CA were more effective antioxidants than BO and MA because they contain hydroxyl groups at positions 2 and 4 of ring A instead of the methoxyl group that both BO and MA have at the same ring positions. Such a hypothesis is consistent with reported positive correlations between the number of hydroxyl groups in the aromatic rings of phenolic acids and the antioxidative and antiradical activity of these compounds (Sroka and Cisowski 2003). The lower reactivity of AT compared to its homologue CA, which has chlorine at position 5, may be due to inductive electron withdrawal of this atom over the *o*- and *p*-phenolic groups of ring A, which may propitiate a higher hydrogen-donating ability of the OH group in the *p*-position of the CA molecule (Burton et al. 1985).

Compared to the common cellularly soluble antioxidant AA, which we used as positive control in the $DPPH^{\bullet}$ experiments, all of the tested phenolics had a rather slow antiradical activity. The reaction between $DPPH^{\bullet}$ and AA reached its plateau within a few minutes compared to over 6 h for all the phenolics (data not shown). Consequently, even the most reactive of the tested phenolics (ARP: 0.11–0.59, Table 2) had a much lower antiradical power than AA (ARP=3.70). According to Brand-Williams et al. (1995), other vascular plant phenolics showed even higher ARP values; e.g., caffeic (11.0) and gallic acids (12.5). However, some plant phenolics reported by the same authors had similar or lower ARP values than the lichen phenolics tested herein; e.g., coumarin, vanillin, and vanillic acid (0.02, 0.05, and 0.17, respectively).

The location of phenolics in the lichen body is a constant feature used by lichenologists as a taxonomical character. Its biological importance was observed in the context of protection from harmful light irradiation (Fahselt 1994). Interestingly, the most reactive phenolics of *P. stuppeum* (CA and AT) and *R. asahinae* (US) are located at the thallus cortex (Culberson and Culberson 1976; Nash III and Elix 2001), which is the thallus region in contact with the surrounding air, whereas the less reactive SA, BO, and MA are medullary and thus less directly exposed. Such reactivity distribution suggests that lichen phenolics, at least those most reactive and cortical, provide some degree of protection against environmental oxidants by limiting their entrance into more internal tissues and the vital cell interior. Reactions between secondary metabolites and O_3 at the lichen surface are likely to occur, but this needs experimental demonstration. Some authors suggest that reactions between trace atmospheric gases and organic compounds on solid or liquid surfaces could be biologically important because they are faster than previously thought (e.g., Donaldson et al. 2005). For instance, ozonolysis of complex organic compounds such as polyaromatic hydrocarbons is documented to occur at the surface of atmospheric aerosol particles and known to be enhanced by low air humidity (Pöschl et al. 2001). Thus, in contrast with pollutants requiring the presence of water or metabolically active cells or tissues to cause injury, O_3 can be expected to degrade biological compounds on dry surfaces. That could be of great importance for organisms growing under O_3 stress, as this pollutant reaches its maximum ambient air concentration at the driest hours of the day. It could be hypothesized that the most reactive cortical phenolics of lichens degrade the most during the midday-early afternoon hours, when the air is driest and these organisms less metabolically active.

Cocchietto et al. (2002) stated that the biological role of lichen substances is strictly species-specific. Our results confirm this view. For instance, by simply summing up the reactivity constants or ARP values in Table 2, the air-pollution sensitive *P. stuppeum* would have a higher potential antioxidant capacity because of phenolics than the resistant *R. asahinae*, which would be a paradox. Such an apparently contradictory result is explained

in the following section once the reactivity rates are combined with the field contents of phenolics in those lichens.

Phenolic Contents in Field Samples Table 3 compares field contents of individual and total quantified phenolics (TQP) in the studied lichens. For *R. asahinae*, TQP was on average 9.2% of the lichen dry weight at the control site, increasing near Mexico City to 9.7, 10.5, and 13.5% at Desierto de los Leones, Ajusco Volcano, and Contreras, respectively. For *P. stuppeum*, TQP represented 7.6% of the lichen dry weight at the control site and increased near the city to 9.1 and 9.8% at Desierto de los Leones and Contreras, respectively. Except for the tolerant *R. asahinae* at Desierto de los Leones, where it had similar TQP values to the control site, the increases near the city were highly significant ($P<0.001$, Table 3), and they can be interpreted as a generalized lichen response to the polluted conditions. However, this still does not explain yet why at the polluted sites *R. asahinae* is dominant, and *P. stuppeum* is severely reduced.

On an individual compound basis, the less reactive and medullary compounds of each species (BO and SA) were more abundant by an order of magnitude than the most reactive, cortical ones (AT, CA, and US, Table 3). This negative correlation between compound abundance and reactivity was nonlinear (Fig. 6). Accordingly, for the studied lichens, it can be established that under natural conditions phenolic degradation increased with reactivity. The site comparisons of individual phenolics had more complex patterns, but they allow one to identify which compounds were responsible for the TQP increases near Mexico City (Table 3). Only BO and US (*R. asahinae*) and SA (*P. stuppeum*) increased consistently near the city; AT and CA (*P. stuppeum*) decreased. MA (*R. asahinae*) had similar levels to the control site (except at Contreras where it increased). The highest and most consistent proportional increase was for US, the most reactive phenolic of *R. asahinae*, which doubled its concentrations near the city. The less reactive and largest contributor to TQP for *R. asahinae*, BO, increased at Ajusco (11%) and Contreras (43%) but not at Desierto de los Leones, which explains why this species did not differ there from the control site in TQP levels. For *P. stuppeum*, only SA increased significantly at Desierto de los Leones (26%) and Contreras (35%); whereas CA, the most reactive phenolic, decreased, respectively, by

Table 3 Mean concentrations (μg/mg of lichen dry weight)[a] of phenolic compounds of two epiphytic lichens from fir forests near Mexico City and the control site (Chico)

Lichen	Compound	Chico	D. Leones	Ajusco V.	Contreras
R. asahinae	US	5.7±0.82[b]	11.0±1.18***	11.8±2.52***	12.0±1.36***
	MA	16.1±1.14	16.9±1.41	15.8±1.46	23.1±1.16***
	BO	70.1±3.83	69.0±3.94	77.6±5.13**	100.2±3.78***
	TQP	91.9±4.72	96.9±5.27	105.2±8.42***	135.3±5.11***
P. stuppeum	CA	4.2±0.30	3.3±0.28***	ND	3.8±0.19**
	AT	6.7±0.38	5.1±0.33***	ND	6.4±0.25
	SA	65.3±3.10	82.4±5.86***	ND	88.2±3.37***
	TQP	76.3±3.16	90.8±6.05***	ND	98.4±3.34***

TQP Total quantified phenolics, *ND* not determined

*$P<0.05$

**$P<0.01$

***$P<0.001$

[a] Significant differences respect to the control site (EL CHICO) from Dunnett test after nested ANOVA

[b] 95% confidence interval, $N=27$

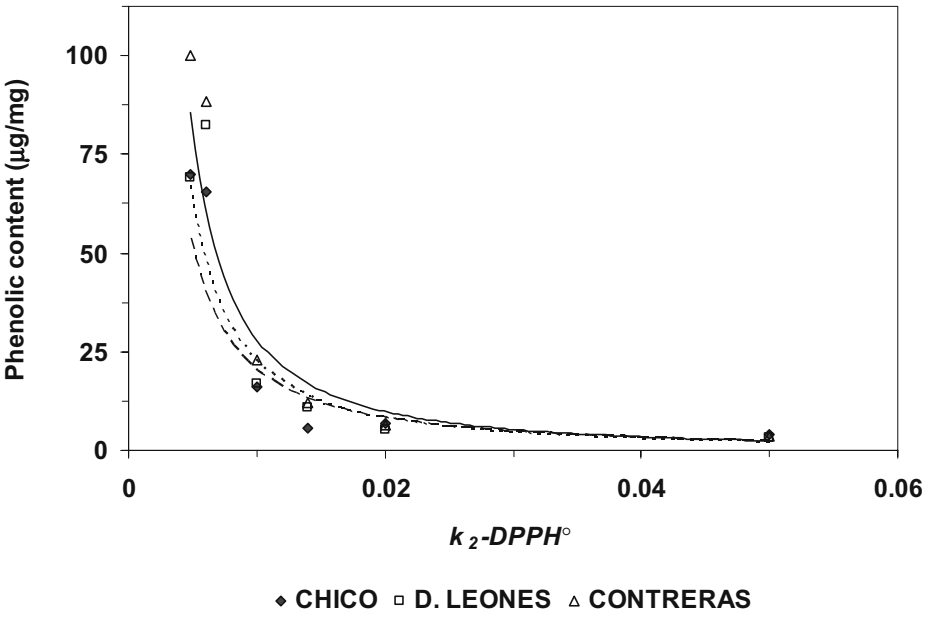


Fig. 6 Phenolic concentrations in field samples of *P. stuppeum* and *R. asahinae* vs free radical scavenging rates (k_2-DPPH$^{\bullet}$). This plot included all six phenolics evaluated in this study. *Lines* fitted as power functions. R^2 values: El Chico (control site), 0.82; Desierto de los Leones, 0.90; Contreras, 0.92

13 and 31% at the same sites. Finally, AT, the second most reactive phenolic of *P. stuppeum*, also decreased (35%) at Desierto de Los Leones, but it did not differ from control values at Contreras. Thus, the TQP increase for this latter species at the polluted sites is attributable to extra production of SA, its less reactive medullary compound.

The potential meaning of the differences in phenolic contents for the tolerance of local lichens toward chronic oxidative air pollution was approximated by weighting the amount of each compound with its anti-free radical capacity: $k_{2\text{-DPPH}}{}^{\bullet}$ [LP]. This product represents a measure of the partial contribution of each phenolic to the total PAC provided by the lichen substances. The $k_{2\text{-DPPH}}{}^{\bullet}$ was used as weighting factor instead of $k_{2\text{-O3}}$ because this latter constant could not be determined for CA (Table 2). Nevertheless, because both constants lead to the same reactivity ranking for the most reactive phenolics and because weighting contents by another constant would not introduce extra variability into the data matrix, similar results could be expected from using either constant. Table 4 shows the site comparisons by lichen PAC values. The tolerant *R. asahinae* increased significantly its total antioxidative capacity ($P<0.001$) at all sites near the city: 13% at Desierto de los Leones, 20% at Ajusco, and 52% at Contreras. The significant PAC increase for this tolerant species at Desierto de los Leones should be emphasized because its TQP did not differ from control values (Table 3). In contrast, the significant increase in TQP for the sensitive *P. stuppeum* at this site did not suffice to elevate its PAC, which was due to degradation of CA and AT. Thus, the potential antioxidative capacity of *P. stuppeum* at Desierto de los Leones depended on its less reactive SA. At Contreras, this species had a PAC greater than at the control site, which was also due to increased concentrations of SA.

Fig. 7 summarizes how these two lichen species respond to the Mexico City environs by changing their phenolic profiles in a sharply different manner. This figure plots the ratios

Table 4 Mean potential antioxidant capacity[a] (PAC) provided by lichen phenolics compounds of two epiphytic lichens from fir forests near Mexico City and a control site (El Chico National Park)

Lichen	Compound	El Chico	D. Leones	Ajusco V.	Contreras
R. asahinae	US	0.08±0.011[b]	0.15±0.017***	0.17±0.035***	0.17±0.019***
	MA	0.16±0.011	0.17±0.014	0.16±0.015	0.23±0.012***
	BO	0.34±0.018	0.33±0.019	0.37±0.025**	0.48±0.018***
	PAC	0.58±0.031	0.65±0.037***	0.70±0.068***	0.88±0.036***
P. stuppeum	CA	0.21±0.015	0.16±0.014***	ND	0.19±0.009*
	AT	0.13±0.008	0.10±0.007***	ND	0.13±0.005
	SA	0.39±0.019	0.49±0.035***	ND	0.53±0.020***
	PAC	0.74±0.023	0.76±0.043	ND	0.85±0.021***

ND Not determined

*$P<0.05$

**$P<0.01$

***$P<0.001$

[a] Significant differences respect to the control site (EL CHICO) from Dunnett test after nested ANOVA

[b] Confidence interval (±95%, $N=27$)

between concentrations in lichen tissue of the more reactive (cortical) and the less reactive (medullary) phenolics (MRP/LRP). The control site ratios can be interpreted as an average cortex-to-medulla phenolic reactivity balance when the lichens grow under low oxidative stress. At the polluted forests, this ratio increased by 45–96% in tolerant *R. asahinae* and decreased by 35–38% for the sensitive *P. stuppeum*. It could be hypothesized that these ratios reflect the degree of oxidative air pollution stress at a given site. If this is correct,

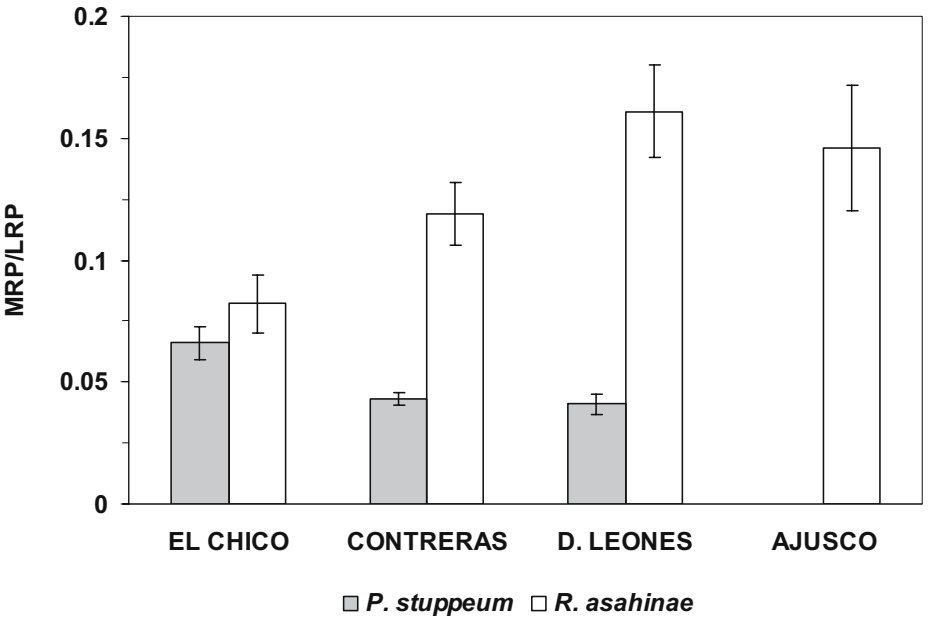


Fig. 7 Mean concentration ratio of the most reactive cortical phenolic (*MRP*) to the less reactive medullary phenolic (*LRP*) for field samples of *P. stuppeum* (CA/SA) and *R. asahinae* (US/BO) growing in fir forests near Mexico City. *Error bars* represent the 95% confidence intervals, $N=27$

Contreras would be the least stressed of the studied sites near the city. Both lichens had their highest absolute increases in phenolic contents at this site, which is probably an exacerbated response from still healthy samples. Levels of AT (*P. stuppeum*) at Contreras were similar to the control site, indicating still balanced biosynthesis and degradation rates for this compound. This hypothesis needs testing by further studies relating site oxidant air pollution levels with lichen phenolic profiles. It should be considered that the antioxidant protection of lichens provided by phenolics is only partial because they also contain other important apoplastic and symplastic antioxidative protection systems (Silberstein et al. 1996; Kranner et al. 2005).

These results showed that lichen secondary metabolites reflect the long-term exposure to oxidant air pollution near Mexico City. They seem to explain partially why *R. asahinae* currently dominates the severely simplified epiphytic lichen communities of the fir forests near Mexico City (Zambrano and Nash III 2000). They also add support for a lichen air-pollution protective mechanism based on secondary metabolites. Such a mechanism may not be universal to lichens as it depends on species specific strategies to modify the balance between phenolic degradation and biosynthesis, as reflected by their ratio between highly reactive and less reactive compounds. The ability of each species to modify these ratios may ultimately prove to be critical for lichen survival in forests severely affected by oxidative air pollution.

In summary, we can state that the six lichen secondary metabolites evaluated in this study showed differential antioxidant and free antiradical activity when mixed, respectively, with O_3 or $DPPH^{\bullet}$ solutions. Although this does not show unequivocally that lichen substances respond under natural conditions to O_3, the comparison of major phenolics between these two species with contrasting tolerance to air pollution from Mexico City does provide support for such a possibility. First, the phenolics with the greatest antioxidant activity, US from *R. asahinae* and CA and AT from *P. stuppeum*, were those deposited on the cortical hyphae, which are the lichen cells most exposed to the atmosphere. This finding, together with the available evidence on surface ozonolysis of complex organic compounds from other experimental systems, suggests that these phenolics likely react with O_3 and thus provide some degree of antioxidative protection under field conditions. Second, the phenolic profiles of the air-pollution tolerant *R. asahinae* and the sensitive *P. stuppeum* differed quantitatively between the chronically polluted and unpolluted forests around Mexico City. Both species had higher TQP near the city, but only *R. asahinae* had higher PAC values in the near-urban sites. The high PAC values in *R. asahinae* were traced to substantial increases in US, the most reactive phenolic of this species. In contrast, the air pollution-sensitive *P. stuppeum* showed significant degradation of CA, its most protective phenolic at the same sites. Together, these results support the hypothesis that lichen phenolics contribute to the antioxidant defenses of these organisms in places affected by chronic oxidative air pollution. The research approach of combining lichen phenolic contents with antioxidative measurements revealed aspects of the differential importance of these compounds, which otherwise may have not been captured. Because lichens are also protected by other apoplastic and symplastic antioxidative systems, to fully assess the use of lichen phenolics as biomarkers for oxidative air pollution, other experiments are needed, such as controlled fumigations with O_3 and observational studies correlating field phenolic contents with air pollution levels.

Acknowledgments This study was supported by the IMP Security and Environmental Research Program. We thank Dr. Luis Zamudio, Corrosion Laboratory, IMP, for doing the NMR analyses of the lichen phenolic isolates, and Dr. Mark Fenn, with the Pacific Southwest Research Station (USDA Forest Service), for improving the English.

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found in all orders of demosponges (McClintock and Baker 2001), a large number of which have potential for development into bioproducts (Sipkema et al. 2005; Paul et al. 2006). The temperate marine sponge *Haliclona* sp. (Demospongiae; Haplosclerida; Chalinidae) has attracted considerable attention because of its production of the potent cytotoxic natural product salicylihalamide A (Fig. 1). Salicylihalamide A represents a class of compounds that has a unique chemical scaffold different from that of known antitumor compounds (Erickson et al. 1997). Its mode of action is via the inhibition of mammalian vacuole ATPase activity, which is implicated in pathological processes such as osteoporosis, renal disease, HIV infection, and tumor metastasis (Boyd et al. 2001). Salicylihalamide A is currently undergoing preclinical trials; however, its success as a drug is hindered by lack of a reliable supply (Newman and Cragg 2004). Whereas salicylihalamide A has been synthesized (Wu et al. 2000), this is currently not an economically viable option for supply. The greatest potential for securing commercial quantities of salicylihalamide A is through harvesting either wild or cultured biomass (Munro et al. 1999), highlighting the importance of developing an understanding of the natural concentrations of the compound (Munro et al. 1999).

New populations of *Haliclona* sp. have been discovered since the sponge was initially collected in 1989 at Rottnest Island (Erickson et al. 1997). Closer inspection of these new populations has resulted in the identification of two morphologies that have similar spicule and skeletal characteristics (unpublished data). The first morphology (green *Haliclona*) has a deep green coloration, an amorphous shape, and varying numbers of oscules. The oscules are chimney-like in shape and extend outwards at least 20 mm from the main sponge. The sponge mesohyl is open with large internal canals present. The second morphology (brown *Haliclona*) has a brown coloration, a mound shaped body, and small, slightly raised oscules that extend less than 5 mm above the surface. The oscules are numerous, and the sponge has a compact mesohyl with few internal canals. Closer inspection of the original voucher sample showed that salicylihalamide A was isolated from the green morphology. The brown morphology has not yet been examined. Understanding the natural concentrations of secondary metabolites provides information on both the biology and ecology of the source organism, such as the relationships between populations and individuals (Turon et al. 1996; Lopez-Legentil et al. 2005), the physiology of the source organism (Turon et al. 1996; Duckworth and Battershill 2001), and environmental influences (Thompson et al. 1987; Maida et al. 1993; Page et al. 2005). Limited ecological information exists for both morphologies of *Haliclona* sp., with most literature restricted to the chemistry of the green *Haliclona*.

Spatial variation in concentrations of secondary metabolites has been documented in several cnidarians (Harvell et al. 1993; Maida et al. 1993; Kelman et al. 2000; Marti et al. 2005), bryozoans (Mendola 2003; Marti et al. 2005), tunicates (Lopez-Legentil et al. 2005; Marti et al. 2005), and sponges (Thompson et al. 1987; Becerro et al. 1995; Page et al. 2005). Fewer studies have examined temporal variation (Turon et al. 1996; Duckworth and Battershill 2001; Marti et al. 2005; Page et al. 2005). Chemical variability has been attributed to several factors such as hydrodynamic characteristics (Duckworth et al. 2004;

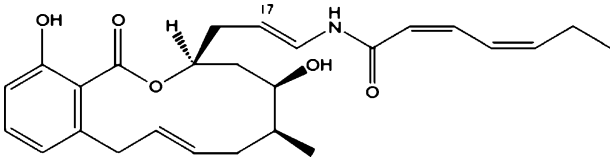


Fig. 1 Chemical structure of the bioactive compound salicylihalamide A

Page et al. 2005), depth (Thompson et al. 1987; Harvell et al. 1993; Page et al. 2005), competition (Maida et al. 1993; Turon et al. 1996), size (Maida et al. 1993; Becerro et al. 1995), and habitat (Becerro et al. 1995).

The primary goal here was to determine whether both morphologies of *Haliclona* sp. produce salicylihalamide A. Secondary to this was to describe the spatial and temporal patterns of salicylihalamide A production in the recently discovered *Haliclona* sp. populations at Hamelin Bay, Jurien Bay, and Bremer Bay, and the ecological implications. This is the first study of the variability of a bioactive compound in a marine sponge from the west coast of Australia.

Materials and Methods

Collection Collections were made haphazardly by SCUBA at each of the four locations (Bremer Bay—34.45°S, 119.38°E; Hamelin Bay—34.20°S, 115.04°E; Rottnest Island—32.00°S, 115.52°E; and Jurien Bay—30.275°S, 115.02°E) along the south western coast of Western Australia (Fig. 2) during the Australian summer (December–February). Winter (June–August) samples were collected only from Bremer Bay because of unfavorable diving conditions at the three other locations. Sponge size was determined before sampling by using an underwater stereo camera to capture a series of stereo images of each sponge (Abdo et al. 2006b). Each set of images was processed in the laboratory to obtain a volume

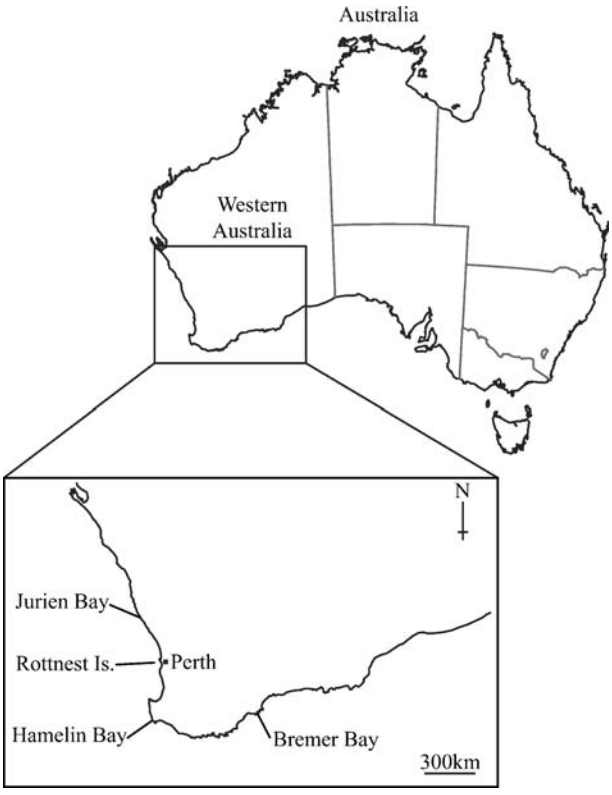


Fig. 2 Study locations along the west coast of Australia. Initially, both morphologies were only known to occur at Rottnest Island but have been found now at Jurien Bay in the north and Hamelin Bay and Bremer Bay to the south. Sponge distribution covers approximately 1,200 km of coastline

estimate for each sponge (Abdo et al. 2006b). Samples (approximately 9 cm^3 in size; N=5, except Rottnest Island where N=6) of both the green and brown morphologies were excised and placed in individual vials and frozen at −20°C. The depth of each sponge, the number of necrotic areas, and the water temperature were also recorded.

Chemical Extraction and Analysis The concentration of salicylihalamide A was determined by using high performance liquid chromatography (HPLC) coupled with ultra violet (UV) detection and mass spectrometry (MS). Each frozen sample (approximately 8 cm^3) was cut into small pieces, placed within preweighed and labeled vials, and freeze-dried for 48 h. The dry weight was recorded (883.29±31.97 mg [mean±s.e.]), and each sample was extracted exhaustively ×3 by addition of 10 ml of ethanol and sonicated for 30 min during each extraction. The supernatant was filtered under vacuum, concentrated in vacuo, freeze-dried, and weighed (67.63±3.45 mg [mean±s.e.]). Samples were dissolved in methanol to give a concentration of 10 mg ml^{-1}. A 160-μl aliquot of each sample was combined with 40 μl (equivalent to 0.5 mg ml^{-1}) of the internal standard, coumarin ($C_9H_6O_2$). For each sample, 40 μl (equivalent to 8 μg μl^{-1} of crude extract) were injected onto a Gemini 3 μ C18 110 Å 50×4.6-mm column (25°C) at a flow rate of 1 ml min^{-1} with a gradient of 50% acetonitrile (ACN): water to 100% ACN over 40 min by using an Agilent 1100 HPLC system (comprising a degasser, binary pump, PDA [for ultra violet detection] and a Gilson 215 Liquid Handler auto sampler/collector). The eluant was directed to a Bruker Daltonics Esquire3000plus mass spectrometer with an Apollo source operating at 40 eV to measure low resolution mass spectral data.

The concentration of salicylihalamide A was quantified by calculating the integral of the peak area at the retention time of 12 min and λ=285 nm (in relation to the integral of the coumarin peak area at 5 min and λ=285 nm) for each sample and converted to a concentration (μg g^{-1} of dry weight) by reference to a standard curve.

Data Analysis Pearson's correlation coefficient (r; Zar 1999) was used to examine correlations between the concentration of salicylihalamide A and water temperature, size, and depth of the sponge. Correlations between water temperature, sponge size, and depth were also examined to ensure that each variable did not influence the other.

The influence of any significant correlations of water temperature, depth, and sponge size with salicylihalamide A concentration was removed with a one-way analysis of covariance (ANCOVA; Zar 1999) to determine if variation in salicylihalamide A concentration occurred among locations (fixed factor, four levels). A two sample t-test was used to examine for variation in the concentration of salicylihalamide A between summer and winter (Zar 1999). For all analyses, data were checked for normality and homoscedascity before analysis, and Bonferonni corrections were applied to account for multiple comparisons (Zar 1999). To determine if the health of the sponge influences the concentration of salicylihalamide A, a two sample t-test was used to compare the salicylihalamide A concentration of healthy (necrotic tissue absent) and unhealthy (necrotic tissue present) sponges.

Results

Differences Between Morphologies Salicylihalamide A was produced by all samples of the green *Haliclona* collected from the four locations and during both summer and winter

seasons at Bremer Bay. The mean concentration of salicylihalamide A within green samples was 10.7 μg g^{-1} (±1.1 μg g^{-1}). The mean yield of salicylihalamide A from the crude extract was 2.83% (±0.28%). Salicylihalamide A could not be detected by either UV or MS in any of the brown samples collected from any location or season (Fig. 3).

Temperature, Volume, and Depth Correlations Water temperature showed an increasing trend in green *Haliclona* from southern to northern locations (Bremer Bay 19.5°C [±0.3°C]< Hamelin Bay 20.1°C [±0.2°C]<Rottnest Island 21.2°C [±0.5°C]<Jurien Bay 22.2°C [±0.4°C]). Salicylihalamide A concentration showed a large, negative correlation (r=−0.703, $P_{\alpha=0.017} < 0.01$) to water temperature in summer (Fig. 4). However, no significant correlation was observed between water temperature and sponge size (r=−0.396, $P_{\alpha=0.017} = 0.075$) or water temperature and sponge depth (r=0.383, $P_{\alpha=0.017} = 0.087$). Interestingly, the difference in mean concentration in the summer between Bremer Bay and Jurien Bay was 5.0 μg g^{-1} with an associated 3°C temperature difference, but the seasonal difference in concentration (5.3 μg g^{-1}) at Bremer Bay was associated with a 1°C temperature change.

Green *Haliclona* sponges ranged in size from 180 to 4,287.5 cm^{-3} with no correlation between sponge volume and salicylihalamide A concentration (r=−0.358, $P_{\alpha=0.017} = 0.111$). No correlation was found between sponge size and the depth (r=−0.002, $P_{\alpha=0.017} = 0.993$). Sponges were collected from depths ranging from 4 to 18 m, and no correlation was observed between depth and concentration of salicylihalamide A (r=0.234, $P_{\alpha=0.017} = 0.306$).

Spatial and Temporal Differences Variability among individuals within a location ranged from 9.0 to 15.9 μg g^{-1} at Bremer Bay; 6.1 to 15.4 μg g^{-1} at Hamelin Bay; 5.1 to 20.6 μg g^{-1} at Rottnest Island; and 5.0 to 13.8 μg g^{-1} at Jurien Bay. Water temperature was the only covariate utilized in the one-way ANCOVA. Salicylihalamide A concentration was influenced by water temperature (Table 1). After accounting for variation because of water temperature, salicylihalamide A differed significantly among all locations (Table 1 and Fig. 5a) except for Hamelin Bay and Bremer Bay (Table 2).

A significant difference (t=3.18, df=10, $P_{\alpha=0.025} = 0.010$) in the concentration of salicylihalamide A was also found between summer (ranging from 9.0 to 16 μg g^{-1}) and winter (ranging from 4.6 to 12.7 μg g^{-1}) at Bremer Bay (Fig. 5b).

Health Influences Only five green *Haliclona* individuals had visible signs of necrosis, and no significant difference (t=0.75, df=19, P=0.462) was observed between healthy and unhealthy sponges.

Discussion

Green *Haliclona* individuals contained salicylihalamide A at all locations and in both seasons at Bremer Bay. The yield from crude extracts in this study (2.83%) was comparable to the 2.55% reported by Erickson et al. (1997) in the initial extraction of *Haliclona* sp. Salicylihalamide A was not detected in any of the brown individuals from the same sites. Given that both color morphologies have similar spicule and skeletal characteristics, this difference in chemical profile may be a useful characteristic in taxonomic classification (Thompson et al. 1987).

Salicylihalamide A concentration was significantly influenced by water temperature, thus probably accounting for some variation among locations. The concentration was also significantly higher in summer than winter in sponges from Bremer Bay. This variation of

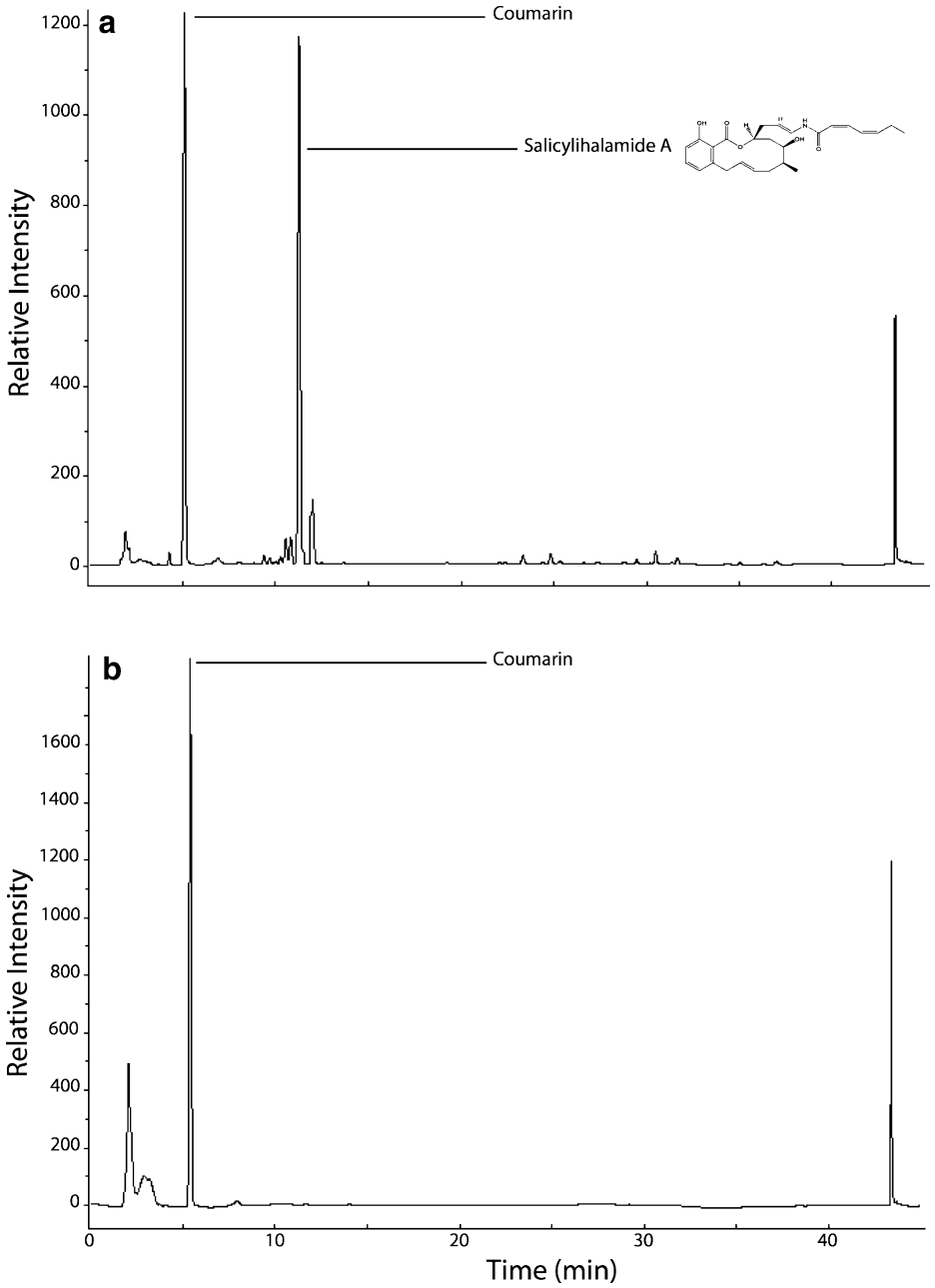


Fig. 3 HPLC chromatographs (λ=285 nm) illustrating the difference in the chemical profiles of **a** green *Haliclona* samples and **b** brown *Haliclona* samples. Salicylihalamide A (at retention time of 12 min) was detected in the green morphology only. Coumarin ($C_9H_6O_2$) was used as an internal standard (at a retention time of 5 min)

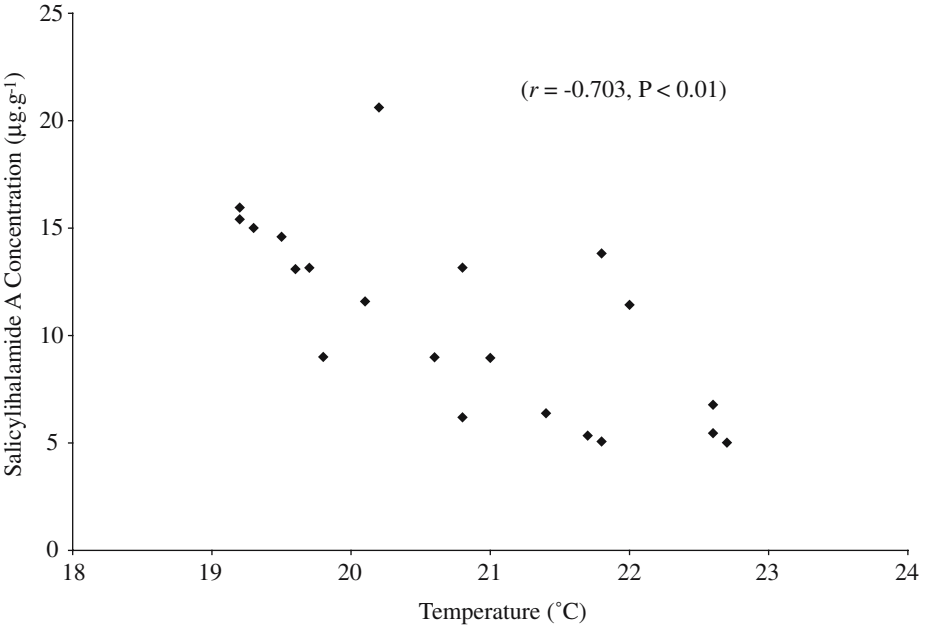


Fig. 4 Correlation ($P<0.017$) between salicylihalamide A concentration and water temperature

salicylihalamide A among locations compares favorably to previous studies where large variability in bioactive compounds has been observed as a result of spatial variation (Thompson et al. 1987; Becerro et al. 1995; Page et al. 2005). Page et al. (2005) found that spatial differences corresponded to a variation in the bioactive compounds produced by *Mycale hentschelli* and that the variation was a result of the environmental differences between locations. This was also evident here, as we found a strong decreasing trend in salicylihalamide A concentration with increasing water temperature. A large environmental gradient occurs along the south west Australian coastline with a 0.5°C rise in water temperature for every degree of latitude (Creswell and Golding 1980), thus resulting in a temperature difference of approximately 3°C between the most northern (Jurien Bay) and southern (Bremer Bay) locations in this study.

Whereas water temperature is an important contributor in the production of salicylihalamide A, other biological or physiological factors, however, also appear to be influencing its production. Salicylihalamide A concentration differed among locations even when the influence of water temperature was removed. The difference in mean salicylihalamide A

Table 1 One-way ANCOVA for the effect of location (among Bremer Bay, Hamelin Bay, Rottnest Island, and Jurien Bay) with temperature as a covariate on the concentration of salicylihalamide A in green *Haliclona* sp.

Source	*df*	SS	MS	*F*	*P*
Location	3	160.661	53.554	22.070	**<0.001**
Temperature	1	286.772	286.772	118.170	**<0.001**
Error	16	38.830	1.472		

Bold values indicate significant result at Bonferroni corrected $\alpha=0.025$.

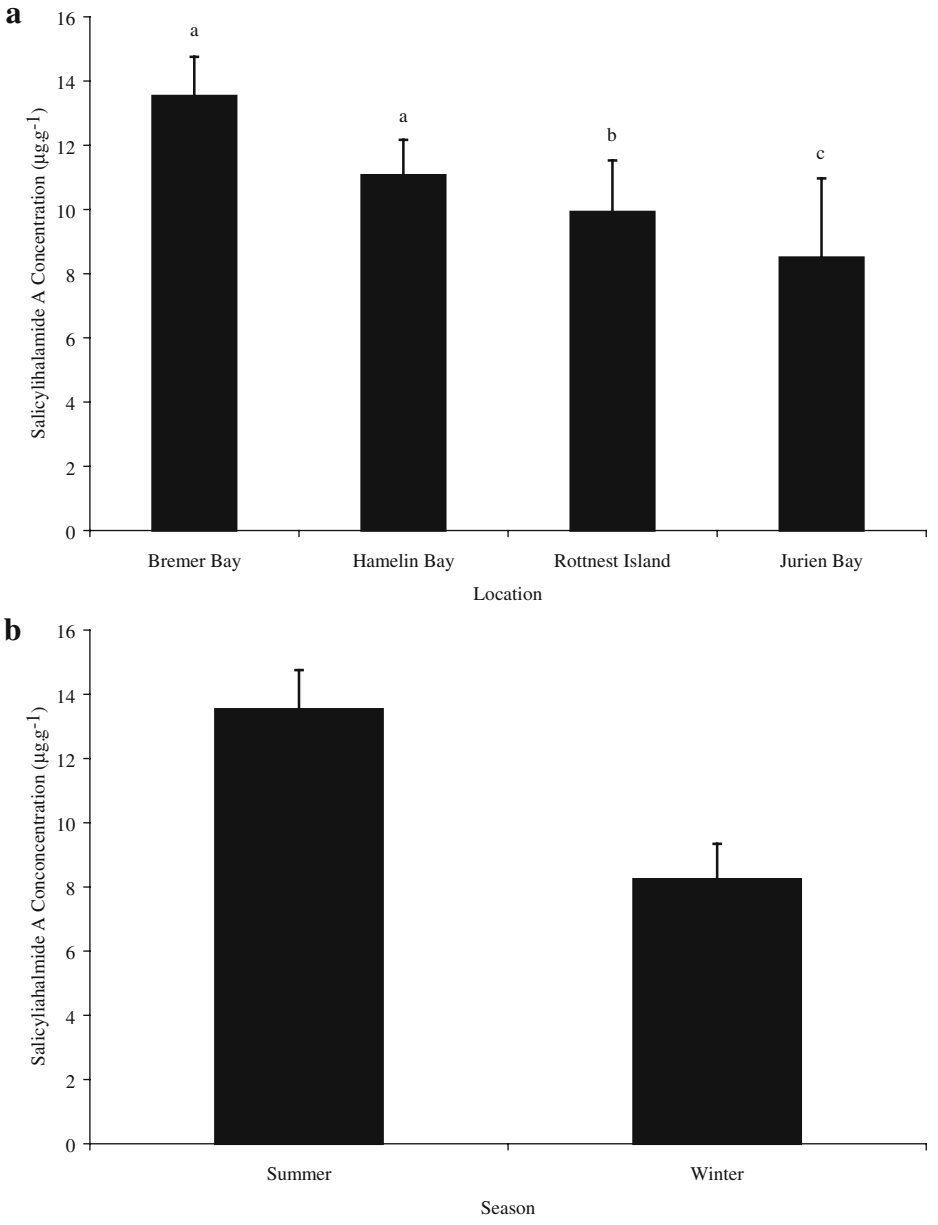


Fig. 5 Difference in mean salicylihalamide A concentration in green *Haliclona* **a** among locations and **b** between summer and winter at Bremer Bay. *Letters* indicate where a significant difference ($P<0.05$) among locations was observed with ANCOVA

concentration between Bremer Bay and Jurien Bay during summer (~5.3 µg g^{-1}) was similar to the difference in mean concentration between seasons at Bremer Bay (~5 µg g^{-1}). However, the difference in water temperature between seasons at Bremer Bay is only a third of the temperature difference between locations during summer (1 vs. 3°C).

Table 2 Post hoc pairwise (Tukey) comparisons among locations (Bremer Bay, Hamelin Bay, Rottnest Island, and Jurien Bay)

Comparison	*t*	*P*
BB→HB	2.181	0.170
BB→RI	6.313	**<0.001**
BB→JB	7.695	**<0.001**
HB→RI	6.053	**<0.001**
HB→JB	8.039	**<0.001**
RI→JB	6.311	**<0.001**

Bold values indicate significant result at $\alpha=0.05$.

BB Bremer Bay, *HB* Hamelin Bay, *RI* Rottnest Island, *JB* Jurien Bay

Organism size influences the level of bioactivity in sponges (Becerro et al. 1995), with smaller individuals having higher levels of bioactivity presumably as a result of their need for a competitive advantage in obtaining space (Becerro et al. 1995). Becerro et al. (1995) suggested that the sponge *Crambe crambe* may have reached a stand-off with its competitors by reaching an optimal area–perimeter relationship that results in a lower energetic demand for border defense (Becerro et al. 1995; and references within). The production of salicylihalamide A did not correlate to the size of the sponge.

Variation of salicylihalamide A concentrations among locations, beyond the influence of water temperature, may be explained by changes in skeletal spicule mass relative to salicylihalamide A concentration (O'Neal and Pawlik 2002). Differences in spicule or skeletal mass because of environmental influences, such as wave exposure, could be driving the variation in salicylihalamide A concentration.

The temporal variation in secondary metabolite production seen in this study is consistent with that observed for other species (Turon et al. 1996; Duckworth and Battershill 2003; Page et al. 2005). Both Page et al. (2005) and Duckworth and Battershill (2003) found that concentration of bioactive compounds was higher in summer than winter. It was suggested that seasonal variation can occur in response to increased fouling of the sponge surface (Duckworth and Battershill 2001) and to an increase in competitors (Turon et al. 1996) during the summer months. The potential ecological role of salicylihalamide A as an antifoulant is considered unlikely as the green *Haliclona* inhabits low light (irradiance<6 $\mu E\ m^{-2}\ s^{-1}$; Abdo et al. 2006a) overhang habitats and has not been observed to be overgrown by algae (personal observation). Depth-related variability in bioactive compounds has also been related to increased fouling by algae (Thompson et al. 1987); however, there was no correlation observed here between the concentration of salicylihalamide A and depth, further indicating that fouling is unlikely to be influencing production.

Haliclona sp. is a brooding sponge that produces well developed parenchymella larvae and is at the peak of its reproductive period during the summer sampling period of this study. Seasonal changes in bioactive compound production have been related to reproduction in octocorals. Coll (1992) showed that the bioactive compounds were present only during the reproductive season and were confined to the reproductive products. However, Turon et al. (1996) proposed that variation in bioactivity of *C. crambe* decreased with increasing reproductive investment. Future research that investigates whether eggs, embryos, and larvae of green *Haliclona* possess salicylihalamide A would help answer this. It would also be beneficial to sample at all locations during winter and at finer temporal scales, as Page et al. (2005) found site-specific temporal differences in the bioactive compounds of *Mycale hentscheli*.

This study has implications for the future development of salicylihalamide A as a pharmaceutical and for management of green *Haliclona* as an exploitable resource. We revealed a high yield population in Bremer Bay during summer. The consistent production of salicylihalamide A in individuals, regardless of depth or sponge size, suggests that this population could be a target for wild harvest. Caution must be taken, however, if considering wild harvesting as a supply avenue, as salicylihalamide A occurs in relatively low levels within the sponge (0.012% g^{-1} tissue dry weight), and large amounts of biomass would be required for commercial supply.

Acknowledgments The authors are indebted to Linda Heap, Caine Delacy, Dave Gull, and Craig Lebens (Bremer Bay Diving Services) for help during field collections. We also thank the anonymous reviewers for improving this manuscript, and Drs. G. Kendrick, J. McDonald, J. Fromont, and S. Whalan for discussion and comments on the research. Funding for this work was provided by a University of Western Australia (UWA) Small Grant to E. H. and C. B. and UWA University Postgraduate Award to D. A. This research was carried out under Fisheries WA Regulation 179 permit and Fisheries WA who are thanked for assistance with permitting.

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concentrations found in the field (0.02–1.2%). Chemical defense in *E. nuttallii* thus plays an ecologically relevant role in this aquatic plant–herbivore system.

Keywords Allelochemicals · Feeding deterrence · Freshwater macrophyte · Herbivory · Invasive plant · Nutritional value · Flavonoids

Introduction

The North American waterweeds *Elodea canadensis* and *Elodea nuttallii* are invasive submersed macrophytes in Europe. *E. nuttallii* Planch. St. John, in particular, is still spreading over Europe and is found in many waterbodies. Waterweeds are competitive, well adapted to a broad array of environmental conditions (Cook and Urmi-König 1985; Simpson 1990), and contain allelochemicals that are active against competing algae and cyanobacteria (Erhard and Gross 2006).

Although *Elodea* species are often preferred food for waterfowl or crayfish (Lodge 1991; van Donk and Otte 1996), they are avoided by many insect herbivores (Newman 1991 and references therein). *E. nuttallii* is avoided by herbivorous larvae of *Acentria ephemerella* (Pyralidae, Lepidoptera, Denis and Schiffermüller), which cause severe damage to other freshwater macrophytes, such as *Potamogeton* spp. or *Myriophyllum spicatum* L. (Gross et al. 2002). Although Berg (1941) reported feeding of *Acentria* larvae on *E. canadensis* in Danish lakes, a study performed at the Finger Lakes, USA, showed that *Acentria* avoided *E. canadensis* when *M. spicatum* was available, and larvae had a higher mortality on the former (Gross et al. 2001). Avoidance of potential host plants and bad performance is often related to low food quality, i.e., poor nutritional quality, the presence of chemical deterrents or toxins.

In general, nitrogen content is considered the major nutrient determining food quality of plants, and many Lepidoptera prefer plants with the highest nitrogen content (White 1993; Newman et al. 1996). Phosphorus may also limit the growth of Lepidoptera larvae (Perkins et al. 2004). Yet, both *E. canadensis* and *E. nuttallii* have high nitrogen and phosphorus contents (Boyd 1968; Jewell 1971; Newman 1991; Kornijow et al. 1995).

While chemical deterrents are well-known to influence host plant selection and performance of herbivores in terrestrial systems, little is known about similar relationships between freshwater plants and their herbivores. Several aquatic angiosperms contain alkaloids (Ostrofsky and Zettler 1986), and watercress tissue is protected by glucosinolates (Newman et al. 1996), both groups of secondary metabolites known as potent feeding deterrents in terrestrial plants. Hydrolyzable polyphenols present in *M. spicatum* possibly affect the growth of *Acentria* larvae (Choi et al. 2002; Walenciak et al. 2002), but larvae still develop into pupae and adults. Some flavonoids seem to deter aquatic herbivores, e.g., deoxyanthocyanin from the aquatic fern *Azolla pinnata* (Cohen et al. 2002). *Elodea* species contain flavone glucosides (Mues 1983), which might act as feeding deterrents, although they were inactive in allelopathic interactions (Erhard and Gross 2006).

The investigation of chemical defenses against herbivores requires the use of artificial diets (Hay et al. 1998). Filter disks and artificial diets supplemented with pure chemicals or plant extracts have been extensively used to feed terrestrial caterpillars such as *Lymantria*, *Spodoptera*, or *Helicoverpa* (Beninger and Abou-Zaid 1997; Calderon et al. 2001; Simmonds and Stevenson 2001). Diets based on agar have been successfully fed to marine omnivores like amphipods, isopods, or sea urchins (Hay et al. 1994; Cruz-Rivera and Hay 2003). So far, only a few studies have addressed feeding deterrents in submersed freshwater plants, and studies with herbivorous freshwater insects are especially rare (see Dorn et al.

2001). Mostly crayfish have been used in bioassays, as these omnivores feed in a wide variety of laboratory assays (Lodge 1991; Bolser et al. 1998; Kubanek et al. 2001). Thus far, nobody has succeeded in rearing *Acentria* or other aquatic lepidoptera on food other than submersed macrophytes. The larvae avoid all common artificial diets (Choi et al. 2002) that would allow adding antifeedants to their food.

Here, we investigated the performance of neonate and older larvae of *Acentria ephemerella* reared on *E. nuttallii* or pondweeds. We determined content of nutrients and flavonoids in pondweeds and in *E. nuttallii* grown under both high or low light to investigate which factor(s) affect this generalist moth larvae. We also developed a bioassay to test the effect of potential allelochemicals, especially flavonoids, on *Acentria*.

Methods and Materials

Plants *E. nuttallii* was collected in summer or autumn in Lower Lake Constance at the Island of Reichenau and also from a small pond at the campus of the University of Konstanz, Germany. Plants were transported to the laboratory on ice and rinsed with tap water to remove algae and debris. They were shock-frozen in liquid nitrogen and stored at −20°C until lyophilization. *Potamogeton perfoliatus* came from Lower, and *Potamogeton lucens* from Upper Lake Constance. The latter was cultivated in aquaria at 16°C, 30–70 μmol m^{-2} sec^{-1} and at a light cycle of L:D 16:8 hr. Leaves of *P. lucens* were harvested immediately before use in feeding assays.

Larvae Diapausing larvae of *Acentria* aged third to fifth instar were collected in autumn 2003 in Lower Lake Constance. Stems of *P. perfoliatus* containing the overwintering larvae were placed into 1.5-l glass jars with dechlorinated tap water and kept at 4°C in a cooling chamber in the dark. Larvae left their hibernicula after 3–5 mo and were then used in bioassays.

Analysis of Plant Nutrient Content Carbon and nitrogen were measured with an elementar analyzer NCS 2500 (CE Instruments/Thermoquest) with atropine sulfate as a standard (58.8% C, 4.0% N, C:N molar ratio 17:1). Phosphorus was determined with an N/P auto-analyzer after acidic persulfate digestion. Due to low biomass, not all plant samples were available for nutrient analysis, resulting in N=2–10.

Analysis of Plant Chemistry Lyophilized and finely ground plants of *E. nuttallii* were extracted twice in 50% (*v*/*v*) aqueous methanol [1 ml solvent per 10 mg plant dry mass (dm)] for 2 hr each. After evaporation of methanol, the concentrated extract was split into two aliquots. One aliquot was further evaporated to dryness and resuspended in ultrapure water (final concentration 100 mg plant dm ml^{-1}), yielding the crude polar extract that was used in larval bioassays. The second aliquot was adjusted to 2.5% aqueous methanol (*v*/*v*) and fractionated by solid phase extraction (SPE) on a C18 cartridge (2 g sorbens, Phenomenex). After washing with 2.5% methanol, the C18 cartridge was stepwise eluted with 10–100% methanol at 10% intervals, yielding 11 fractions, including the wash fraction. Fractions were evaporated to dryness and resuspended in water (fractions 1–6 that eluted with 10–50% methanol) or 50% methanol (fractions 7–11, eluted with 60–100% methanol). HPLC analysis with a reversed phase C18 LiChrospher column (250×4 mm), a linear gradient with 1% aqueous acetic acid and methanol as solvents, and a photodiode array detector set at 345 nm as primary detection wavelength (details in Erhard and Gross, 2005) showed that

fraction 2 (eluted with 10% methanol) contained flavonoids. This fraction was used in feeding assays. Control extracts and fractionations were performed accordingly, using only solvents but no plant material. Concentrations of luteolin-7-*O*-diglucuronide were calculated from a calibration curve [regression equation: luteolin-7-*O*-diglucuronid (μg)=peak area at 345 nm (A)/5.58 (A μg^{-1}), $R^{2\circ}$=0.99] established with a standard of this compound isolated from *Elodea*. As no pure standards of the glucuronids of apigenin and chrysoeriol were available, we quantified those by their peak areas assuming similar extinction coefficients as for the luteolin-diglucuronide.

Purified metabolites were obtained by extracting 15.5 g fresh *E. nuttallii*, which was homogenized in 250 ml of 50% methanol by using a blender and vigorously stirred over night. After filtration under reduced pressure, the resulting pellet was again treated with 250 ml of 50% methanol and stirred for 2 hr. The solvent was removed under reduced pressure, and the remaining residue taken up in 5 ml of 50% methanol. After centrifugation, the crude polar extract was separated by repeated injections on a preparative HPLC (Gilson 321) using a GromSil 120 ODS-4 column (250×4 mm). Separation of the flavonoids was achieved with a linear gradient of 0.5% aqueous formic acid and methanol starting from 25% methanol to 75% methanol over 30 min. The compounds were identified by two-dimensional NMR techniques and by comparison of the ^{13}C NMR data with the literature (Mues 1983).

Preparation of E. nuttallii-Supplemented Leaves We supplemented *P. lucens* leaf squares with crude polar extract or isolated flavonoids from *E. nuttallii*. Leaves from *P. lucens* were dissected in small pieces of approximately 4×4 mm, omitting the midrib. Leaf squares were placed in 24-well tissue culture plates (two pieces per well) and incubated for 24 hr in aqueous extract, the aqueous flavonoid fraction (100 mg plant dm ml^{-1} each), or the respective controls (400 μl per well). These impregnated leaf squares were used in feeding assays.

The success of leaf supplementation was verified by extracting flavonoids from incubated leaf squares not fed to larvae. Leaf squares were frozen in liquid nitrogen directly after incubation in crude polar extract (0 hr) and 24 hr after application to the inner filter (see below). They were freeze–dried, weighed, and extracted twice for 2 hr with 500 μl 50% methanol in a sonication bath. Aliquots of the extracts were analyzed by HPLC, and we determined the concentration of luteolin-7-*O*-diglucuronide per milligram dry *P. lucens* leaf square to evaluate the effectiveness of supplementation.

Feeding Assays

Performance of First Instar Larvae (Experiment I) In summer 2002, we successfully reared in the laboratory adults that oviposited on *P. perfoliatus* in aquaria. Egg clutches were separated, and neonate larvae hatching from a distinct clutch were divided into four groups, each receiving different diets. The first group was fed *E. nuttallii* (EN) from the campus pond, while two other groups were fed for 3 (PF1) or 5 wk (PF2) *P. perfoliatus*, before receiving *E. nuttallii*. The last group (PF3) got exclusively *P. perfoliatus* (*N*=17–34 per group, for details see Table 1). Larvae were raised in 24-well microtiter plates filled with 2 ml dechlorinated tap water. Larvae received fresh food and water biweekly, and growth was measured by their head capsule width after 3, 5, and 7 wk.

Performance of Older Larvae (Experiments II and III) We performed four no-choice feeding assays, two using *E. nuttallii* shoot fragments or *P. lucens* leaves ("fresh plants", experiments IIa and IIb) and two using supplemented leaves (experiments IIIa and IIIb).

Table 1 Numbers of surviving and dead *Acentria* larvae in the different treatments of experiment I after 7 wk

Treatment		Number (*N*)	Survivors	Dead	
Clutch I	EN	18	0	18	a
	PF1	17	3	14	a, b
	PF2	17	5	12	b
	PF3	17	6	11	b
Clutch II	EN	34	3	31	a
	PF1	34	2	32	a
	PF2	33	11	22	b
	PF3	33	11	22	b

Different letters indicate significant differences at the end of the experiments (χ^2-tests, $P<0.05$).

PF1 Reared on *P. perfoliatus* for 3 wk, then fed *E. nuttallii*, *PF2* reared on *P. perfoliatus* for 5 wk, then fed *E. nuttallii*, *PF3* fed *P. perfoliatus* only, *EN*: fed *E. nuttallii* only

Before all assays, larvae were kept individually in 50-mm glass petri dishes filled with 8 ml of dechlorinated tap water. They were reared on *P. lucens* for 1 d at 16°C in dim light (30 μmol m^{-2} s^{-1}, 16:8 L:D) to make sure that all individuals were actively feeding. After measuring their headcapsule width, larvae were equally distributed to the different treatments (N=13–15 per treatment) to avoid uneven initial headcapsule widths between treatments. Each larva was transferred to a new petri dish filled either with tap water (experiment II) or covered with moist filter paper (Macherey & Nagel, MN615) at the bottom (experiment III). In the latter case, we used two filters: a ring, cut from a 55-mm diameter filter, covered the rim of the bottom, whereas a 27-mm diameter filter was placed in the middle of the petri dish to carry the leaf disk.

As light intensity might influence nutrient composition and content of potential allelochemicals, we tested in two independent experiments whether *Acentria* responds differently to *Elodea* grown under high or low light. Control larvae received *P. lucens*. In experiment IIa (N=13 per treatment), *Elodea* plants were collected from two different sites of the university pond. One site was exposed to full sunlight, whereas the other was in the shade and seldom received full sunlight. Midday light intensities varied between 780 and 1,490 μmol m^{-2} s^{-1} at the sun-exposed site and 107–382 μmol m^{-2} s^{-1} at the shaded site (measured directly under the water surface on five summer days). Water temperature was, in general, 16–18°C, but reached up to 21°C in sun-exposed areas.

Experiment IIb was conducted to determine if light intensity and not other site factors affected eventual differences in plant chemistry or nutrients. We established cultures of pond *E. nuttallii* under controlled conditions in a light chamber. One or two cleaned shoots each were planted in 16 1.5-l glass jars filled with Lake Constance sediment and dechlorinated tap water. After 2 d, plants were divided into two groups. Eight jars were covered with black nylon stockings (20% denier yarn; 154±15 μmol m^{-2} s^{-1}, mean±SD), and the others remained uncovered (544±30 μmol m^{-2} s^{-1}). Water temperature varied between 18.4 and 20.1°C. After 2 wk, plants from both treatments were used as food, and *P. lucens* as control (N=15 per treatment).

To test whether chemicals present in *E. nuttallii* affected feeding behavior, growth, and/or survival of the larvae, *Acentria* were fed in two different no-choice assays either with untreated, control-solvent, crude polar extract, or flavonoid fraction treated leaf squares (N=14 per treatment in both assays). Depending on consumption of the leaves, we prepared new leaf squares every 1–3 d. Together with new food, we also changed the inner filter. Over 20 d,

headcapsule width was measured four times. In addition, the feeding behavior of individual larvae and feeding damage on the leaves was assessed. In experiment IIIa, we only discriminated between damaged and undamaged leaf squares. In experiment IIIb, we distinguished five damage categories: no damage, low consumption corresponding to 1–33% of the leaf, medium (34–66%) and high consumption (67–99%), or whole leaf square consumed.

Statistical Analysis All analyses were conducted with the statistical package JMP IN 5.1 (SAS Institute Inc.), Statistica 6.0 (StatSoft, Inc., Tulsa) or with the software "R", version 2.3.0 (Ihaka and Gentleman, 1996). Nutrient concentrations, flavonoid content, and headcapsule widths were analyzed, whenever possible, by analysis of variance (ANOVA) followed by unequal *N* HSD tests. This test is equivalent to the Tukey HSD test, but considers unequal sample sizes. Homogeneity of variances was tested by using Levene's test, and data were considered normally distributed when Kolmogorov–Smirnov tests resulted in $P>0.05$. When transformation failed to correct for heterogeneity of variances, we used Kruskal–Wallis *H* tests. Individual growth of larvae in experiment IIIa was compared by *t*-test (*E. nuttallii* vs solvent control). Mortality and feeding behavior of the larvae were analyzed by χ^2 contingency tests or Fisher's exact test. In experiment IIIb, we determined the relative proportion of every consumption level for each larva. The relative proportion is calculated as the number of observations of the consumption level divided by the sum of all observations. Differences in feeding behavior of larvae ($N=14$) in the five treatments were analyzed separately for each of the five consumption levels by using Kruskal–Wallis *H* tests. If these revealed significant differences ($P<0.05$) between groups, the data were compared by Nemenyi tests ($k=5$ treatments, $N=14$ replicates; difference in rank sums >293.8 for $\alpha=0.05$).

Results

Nutrient Content Carbon concentrations varied slightly between *Elodea* from the field (36.9 and 35.8% in sun-and-shade-exposed plants) and *P. lucens* (38.7%, one-way ANOVA, $F_{2,13}=4.05$, $P=0.043$), yet pairwise differences were not significant (unequal *N* HSD test, $P>0.05$) (Fig. 1). Nitrogen content in *P. lucens* was lower than in *E. nuttallii* collected in shaded areas (2.3 vs 3.3%; unequal *N* HSD, $P=0.031$). Phosphorus content did not differ among treatments in experiment IIa (one-way ANOVA, $F_{2,13}=3.07$, $P=0.081$).

Elodea grown under high and low light in the light chamber had slightly lower carbon contents than *P. lucens* (37.6 and 36.2% vs 40.7%; unequal *N* HSD, $P=0.039$ and 0.002, respectively). Nitrogen content ranged from 3.4 to 3.7% and did not vary among plants. Phosphorus concentrations were higher in *P. lucens* than in low and high light grown *Elodea* (0.49 vs 0.26 and 0.22%; unequal *N* HSD; $P=0.036$ and 0.006, respectively).

Flavonoid Content Diglucuronides of luteolin, apigenin, and chrysoeriol were present in all *Elodea* samples but not in *P. lucens* (Fig. 2). In some, but not all samples of the latter, a compound with a similar absorption spectrum (λ_{max} at 249, 289, and 329 nm) but a longer retention time was present, indicating the presence of another flavonoid. *Elodea* from the field contained more apigenin- and chrysoeriol-7-*O*-diglucuronides than plants cultured in the light chamber (two-way ANOVA, $F_{1,28}=62.09$ for apigenin and 58.93 for chrysoeriol, $P<0.001$), whereas the luteolin glucuronide did not differ ($F_{1,28}=3.37$, $P=0.077$). In both experiments, flavonoid concentrations were independent of light regime ($P>0.7$).

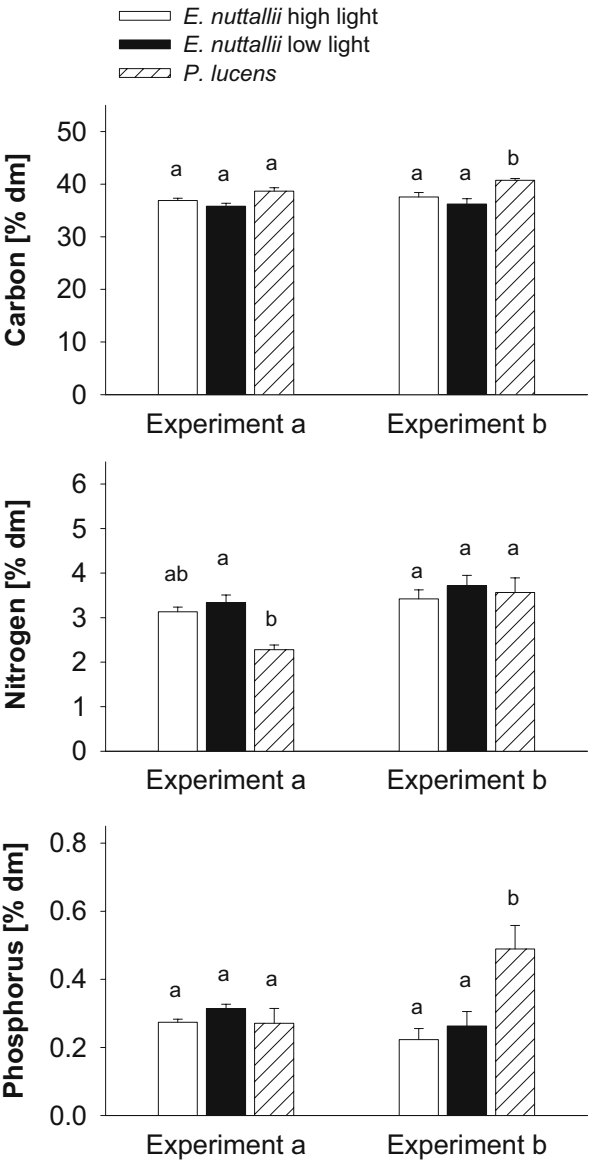


Fig. 1 Nutrient content (% dm) of fresh *P. lucens* and *E. nuttallii* from different light regimes used in growth assays with *Acentria*. In experiment a, *E. nuttallii* was collected in the field. In experiment b, it derived from a laboratory culture. Data represent means+SE (N=2–10). *Different letters* indicate significant differences $P<0.05$

Performance of Neonate Larvae (Experiment I) Neonate larvae of *Acentria* experienced a high mortality (65–100%) during the first weeks after hatching. Significantly more larvae died when fed only *E. nuttallii* compared to those fed first on *P. perfoliatus* for 5 wk and then moved to *E. nuttallii* or those fed only *P. perfoliatus* (Table 1). Furthermore, larvae fed *E. nuttallii* for 7 or 5 wk (EN and PF1) remained smaller than larvae fed most of the time (5 wk) or always on *P. perfoliatus* (PF2 and PF3; unequal N HSD, $P<0.05$ each after two-way ANOVA, clutch I: treatment, $F_{2,59}=3.39$, $P=0.04$; clutch II: treatment, $F_{3,103}=8.67$, $P<0.001$; data not shown).

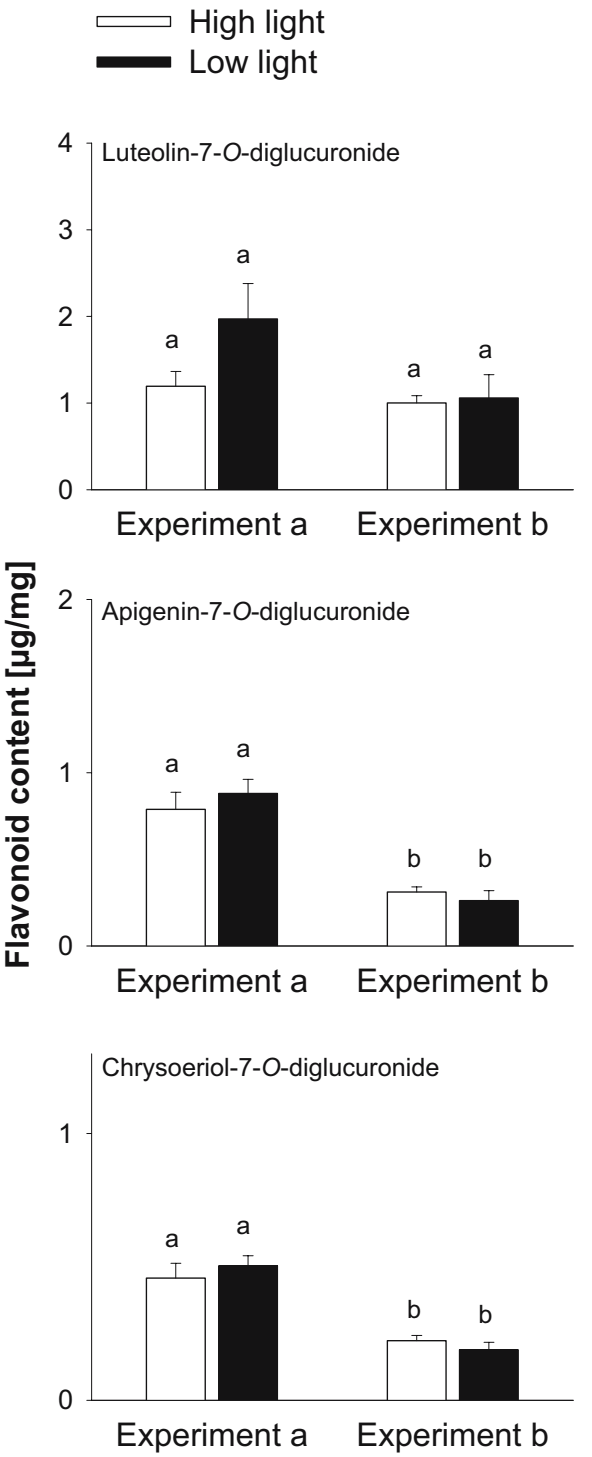


Fig. 2 Content (µg mg^{-1} plant dm) of 7-*O*-diglucuronides of luteolin, apigenin, and chrysoeriol in *E. nuttallii* from different light regimes (high and low light) used in growth experiments with field collected plants (experiment a) and laboratory grown plants (experiment b). Data represent means+SE (N=7–10). *Different letters* indicate significant differences $P<0.001$

Mortality of Older Larvae When reared on fresh plants (experiments IIa and b), none of the larvae fed *P. lucens* died, whereas feeding on *Elodea* grown in high light led to increased mortality (46 and 53%; χ^2-test, P=0.02 and 0.004, respectively; Fig. 3a and b). We observed high fatalities in the first experiment with supplemented leaves (experiment IIIa; Fig. 3c and d), which were mainly caused by escaping larvae. Of the initial 14 larvae, eight fled from the *Elodea* treatment and five from the controls. Four larvae died in the *Elodea* treatment, whereas all control larvae survived, resulting in a significantly increased mortality in the *Elodea* treatment (χ^2=7.337, P=0.007). To prevent escape of larvae in experiment IIIb, we sealed petri dishes with parafilm so that mortality was mainly based on larvae that died in the treatments. Only a few larvae escaped (four from the *Elodea* treatment and one from each of the flavonoid and flavonoid control treatments) due to leaks in the parafilm seal. Similar to experiment IIIa, mortality in the crude extract treatment was marginally increased compared to the other treatments (χ^2=9.333, P=0.053). Flavonoids caused no higher mortality compared to controls (Fig. 3d).

Growth on Elodea Under Different Light Regimes In experiment IIa, larvae fed *P. lucens* increased by 77% in headcapsule width, while those fed sun-exposed and shaded *E. nuttallii* increased by only 18–36%, respectively (Fig. 4a). A two-way ANOVA showed significant food plant and time effects and an interaction of both (plant, $F_{2,163}$=18.672, P<0.001; time, $F_{4,163}$=32.616, P<0.001; plant×time, $F_{8,163}$=3.6, P<0.001).

Similar results were obtained in experiment IIb, where *E. nuttallii* was cultured at low or high light intensity in the laboratory. Both low and high light grown *E. nuttallii* suppressed *Acentria* growth compared to *P. lucens* (Fig. 4b). Here, larvae fed low or high light *E. nuttallii* grew by only 7–15% (both fourth/fifth instar), respectively, and were not significantly different (unequal N HSD, P>0.05). In contrast, larvae reared on *Potamogeton* grew by 39% and were larger than those in both *Elodea* treatments (fifth/sixth instar; $P\leq$0.029). A two-way ANOVA comparing growth after day 12 exhibited significant food plant and time effects but no interaction effect (plant, $F_{2,106}$=37.90, P<0.001; time, $F_{2,106}$=5.05, P<0.001; food×time, $F_{4,106}$=0.32, P=0.867).

Growth of Acentria on Elodea-Supplemented Leaves We compared growth of *Acentria* reared on untreated leaf squares with the growth of larvae fed with leaves incubated in crude polar extract. Starting with day 12, larvae reared on *Elodea*-extract supplemented leaves were smaller than larvae that received control leaf squares (Fig. 4c). Headcapsule width at the beginning of the experiment was 502±54 and 502±51 μm (mean±SE, N=14 each) for treatment and control, respectively. At day 20, larvae reared on untreated leaf squares had reached a mean size of 676±85 μm (N=9), whereas the two remaining larvae fed *Elodea* supplemented leaves had only 557 and 574 μm (N=2). Although these values are outside the 95% confidence interval of the control mean (i.e., 607–745 μm), repeated measures ANOVA (rmANOVA) did not detect any differences between the two groups. As this might be due to the high mortality in the *Elodea* treatment, we compared the individual growth of surviving larvae in the treatments. Headcapsule width of control larvae increased by 176±22 μm (mean±SE), significantly more than that in the *Elodea* treatment (46±21 μm; P=0.028, t-test).

Enrichment of P. lucens with E. nuttallii Extract After incubation in aqueous *E. nuttallii* extract, *P. lucens* leaf squares contained 0.35±0.05% dm (mean±SD, N=3) luteolin-7-*O*-diglucuronide and 0.05±0.004% dm of the other flavonoids (apigenin and chrysoeriol-7-*O*-diglucuronides). When these leaf squares were placed on moist filter paper, most of the

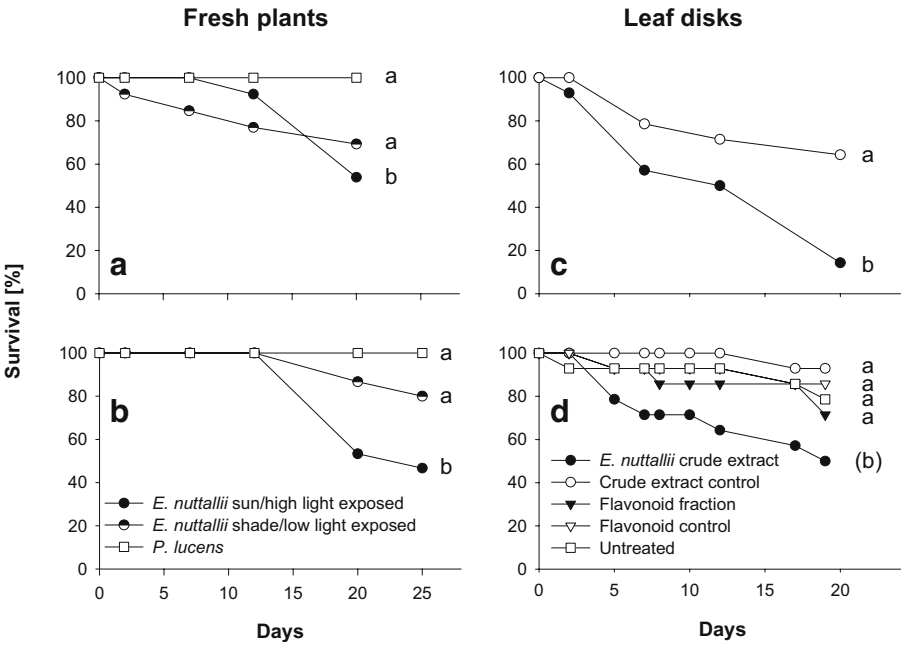


Fig. 3 Survival of *Acentria* on fresh plants (*left panel*) and supplemented *P. lucens* leaf disks (*right panel*) in four independent experiments (initial N=13–15 each). **a** field collected plants, **b** laboratory grown plants, **c** leaf squares supplemented with crude polar extract, **d** leaf squares supplemented with extract fractions as food source. *Letters on the right* indicate significant (P<0.05) groupings among treatments based on χ^2-analyses. *Letters in parentheses* indicate marginal differences (P<0.1)

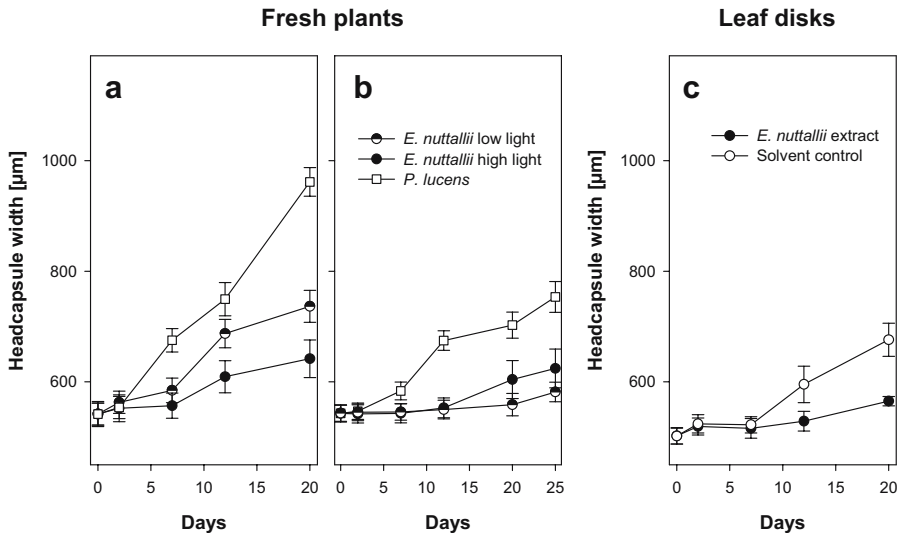


Fig. 4 Growth, measured as headcapsule width (µm) of *Acentria* larvae fed fresh plants from the field (**a**) and from the laboratory (**b**) or supplemented *P. lucens* leaf disks (**c**). N=15 (**a**), 13 (**b**), and 14 (**c**) per treatment at beginning. Data represent means±SE

flavonoids directly diffused into the filter. Only 0.09±0.03% of luteolin and 0.01±0.002% of apigenin/chrysoeriol were found at this time in the leaves, indicating that flavonoids were adsorbed at the surface of *P. lucens*, but were not incorporated into the plant tissue. Amounts detected in the filter paper were 0.8–2.4 and 1.5–2.8 times compared to total leaf squares (luteolin and apigenin/chrysoeriol, respectively). After 24 hr, leaves still contained 0.01±0.007% of the luteolin-diglucuronide, and 0.002% of apigenin/chrysoeriol. At the same time, filter disks had flavonoid contents up to 8.7 (luteolin) and 12.3 times (apigenin/chrysoeriol) higher than the total respective supplemented leaves.

Feeding Behavior of Acentria Larvae fed significantly more often on *P. lucens* soaked in control solvent than on extract treated leaf disks (experiment IIIa, Fisher's exact test, $P<0.001$). In experiment IIIb, we tested whether flavonoids present in *E. nuttallii* influence feeding patterns (Fig. 5). Larvae in this assay exhibited normal feeding behavior that was not different from other assays, but headcapsule widths did not increase at all, even in controls. We observed that larvae were growing, indicated by a visible increase in larval body size. We distinguished the amount of leaf consumed to gain information on larval food uptake. It was striking that larvae did not eat much in all treatments during the first 2 days. Thus, groups did not differ from each other on day 2 (*H*-test, $P=0.458$). After this period, consumption increased in the controls, to a lesser extent in the flavonoid treatment, but not in the crude extract treatment, where until day 10, only two of the offered leaf pieces were damaged (data not shown). In each treatment, except in the crude extract control, some larvae did not eat at all. More larvae showed no consumption in the crude extract and flavonoid treatment ($N=5$ each) than in untreated leaves ($N=2$) and in the flavonoid control ($N=1$). We observed all levels of consumption in every group, but most larvae reared on leaves impregnated with crude polar extract or flavonoids did not feed (86.6±3.7% and 71.1±7.5%, mean±SE, respectively), while total avoidance was less frequent in all three control groups (39.4, 32.5, and 32.4% of untreated, extract, and flavonoid controls, respectively; Nemenyi test $P<0.05$, following *H* test, $H=30.865$, $P<0.001$). In control treatments, larvae consumed significantly more entire leaf pieces (39.6, 41.7, and 49.4%; Nemenyi test $P<0.05$, following *H* test, $H=36.919$, $P<0.001$). A difference in the high consumption level was only visible for the crude extract fed larvae. The proportions of low and moderate consumption ranged between 1.4 and 8.6% and did not differ between groups.

Discussion

Acentria larvae perform better on native pondweed than on the invasive *E. nuttallii*. When reared on suitable food plants, *Acentria* usually has six larval instars until pupation (Gross et al. 2002). Larvae fed *Potamogeton* reached this instar, while those fed *E. nuttallii* had headcapsule widths equivalent to the fourth or fifth instar within the same time span. This indicates that larval development on *Elodea* is delayed. Even if surviving larvae would enter the pupal stage, the prolonged larval development might lead to lower pupal weight, reduced female fecundity, or greater time of exposure to enemies (Price et al. 1980; Haukioja and Neuvonen 1985; Tikkanen et al. 2000).

The impact of *E. nuttallii* on neonate larvae apparently differs from that on older instars after hibernation, which we used in our further experiments. Although there might be exceptions, first instars generally experience higher mortality rates than later instars (Zalucki et al. 2002). Our results with newly hatched larvae show a significant increase in

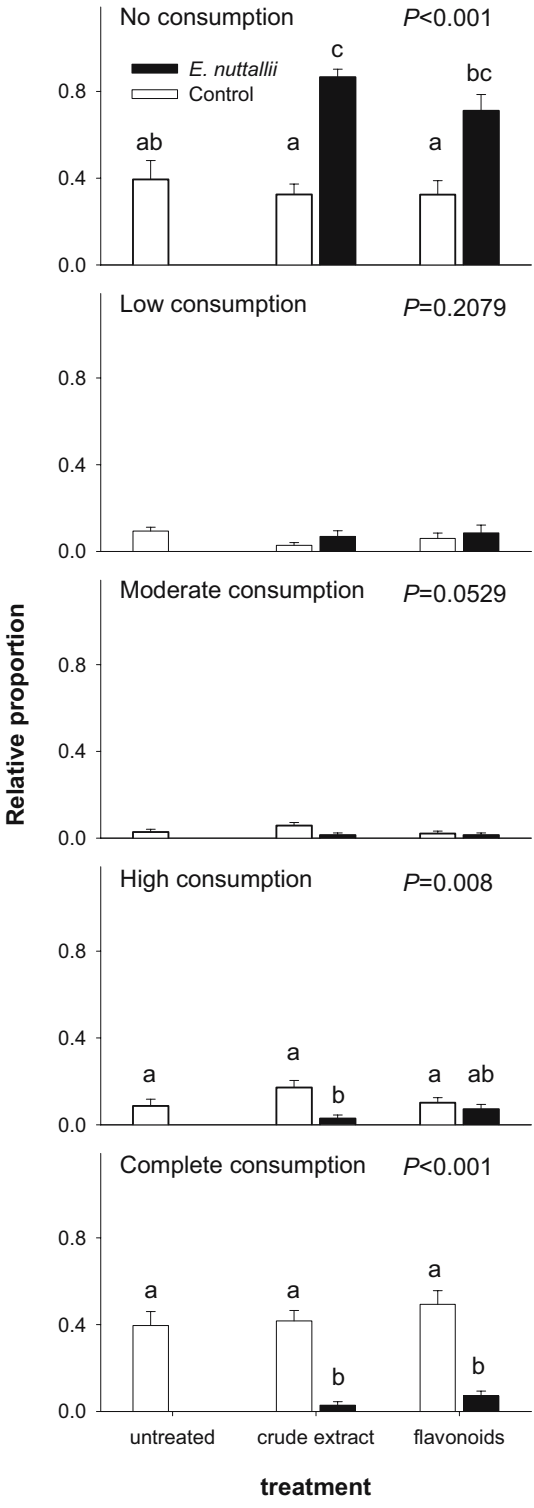


Fig. 5 Proportional contribution [×100%] of different consumption levels among treatments during experiment IIIb. Given are means+SE (N=14 per treatment). *Different letters* indicate significant groupings among treatments (H test followed by Nemenyi test, P<0.05). Levels of feeding damage correspond to 0% (no), 1–33% (low), 34–66% (moderate), 67–99% (high), and 100% (all)

mortality of early instar *Acentria* when fed *E. nuttallii*, ranging between 90 and 100%. In a comparable experiment, *E. canadensis* increased mortality of early instars but only to 50% (Gross et al. 2001). This suggests a more deleterious impact of *E. nuttallii* on the fitness of *Acentria* than *E. canadensis*. A direct comparison of both species in one experiment is needed for final proof.

Structural defenses are most likely not a reason for the poor performance of *Acentria* larvae on *E. nuttallii*, as leaves consist of only two cell layers, and plants were consumed by larvae in our assays. Also, nutritive value did not explain the reduced growth or survivorship of larvae. The average nitrogen content ranged from 3.1 to 3.7% in *E. nuttallii*, comparable to other studies (Newman 1991; Kornijow et al. 1995) and within the range of 1.2 to 4.8% for various submersed angiosperms (Boyd 1968; Jewell 1971; Muztar et al. 1978; Choi et al. 2002).

Cronin and Lodge (2003) measured lower nitrogen levels in freshwater plants grown under high light conditions, but palatability did not differ between treatments. In *M. spicatum*, high light increased nitrogen content (Gross 2003). In our experiments, light had no significant effect on nitrogen content in *Elodea*, and its concentrations did not differ from that in *Potamogeton*. Kornijow et al. (1995) also found low C:N ratios in *E. nuttallii*, and they considered factors other than nutritional value important for herbivores. Also, food plants were never phosphorus limited (Gerloff and Krombholz 1966). Due to the negligible differences in nutrient content in food plants, we consider neither nitrogen nor phosphorus responsible for the bad larval performance.

Our results indicate that allelochemicals in *E. nuttallii* deter feeding by *Acentria* and might inhibit growth of larvae. Crude polar extract and a fraction containing the major three flavonoids from *Elodea* deterred *Acentria* from feeding on *P. lucens* leaves, while untreated or solvent control leaves of *P. lucens* were often completely consumed (Fig. 5). Not many studies have yet investigated chemical defenses in submerged freshwater angiosperms. Many studies have focused on marine macroalgae (Hay 1996; Paul et al. 2006) and emergent or floating-leaved freshwater angiosperms (Bolser and Hay 1998; Dorn et al. 2001; Kubanek et al. 2001; Prusak et al. 2005). These studies often used omnivorous crustaceans as grazers, against which extracts of submersed species have turned out to be inactive (Bolser et al. 1998; Prusak et al. 2005). In contrast, other authors have suggested chemical defenses in submerged freshwater macrophytes against planktivorous *Daphnia*, lepidoptera, and gastropod herbivores based on observed effects and correlations between secondary plant metabolites and feeding activity or changes in behavior (Pennak 1973; Burks et al. 2000; Choi et al. 2002; Cohen et al. 2002; Elger and Lemoine 2005). None of these studies tested isolated chemicals.

The lack of studies establishing evidence for chemical defenses in (submerged) freshwater macrophytes results from methodical problems. Omnivores or shredder herbivores accept various artificial diets (Lodge 1991; Newman et al. 1996; Bolser et al. 1998). The specialized feeding habits of invertebrates challenges the design of suitable artificial diets (Hay et al. 1998). *Acentria* feeds on several species in different freshwater plant families and can thus be regarded as a generalist (Buckingham and Ross 1981). Nevertheless, it consumes exclusively fresh tissue from submerged angiosperms and rejects decaying material or common artificial diets (Choi et al. 2002; D.E. and E.M.G., personal observations).

Acentria larvae did not feed on boiled plant material or other artificial diets (data not shown), and only extract coated fresh leaves could be used in feeding assays. A similar design, in which *Salix* leaves were coated with extracts of several floating-leaved freshwater macrophytes, was used previously in feeding assays with the semiaquatic

lepidopteran *Munroessa gyralis* (Dorn et al. 2001). We tested whether coating of *P. lucens* leaves with *E. nuttallii* extract or fractions would decrease their palatability. The coating of *P. lucens* leaves with *E. nuttallii* extract was not simple. The leaves are thin and do not have a substantial cuticle, which makes them sensitive to organic solvents or wilting. Moist conditions promoted the diffusion of the amphiphilic active compounds in our extracts of *E. nuttallii*. It was necessary to minimize the leaching of extracts into the surrounding medium to make the exposure to active compounds as realistic as possible. The amount of flavonoids enriched in *P. lucens* equaled that of concentrations found in field samples of *E. nuttallii* (0.02–1.2% of plant dm for luteolin-7-*O*-diglucuronide, calculated from values in Fig. 2 and those given in Mues 1983), but flavonoids leached very quickly from the leaves. The inner filter paper prevented the diffusion of the compounds to the outer filter paper, where larvae in *Elodea* treatments spent most of their time. Thus, larvae came in contact with *E. nuttallii* allelochemicals only when they entered the inner filter disk for feeding. Although our method can be further improved, we assume that during feeding on or mining into stems of *E. nuttallii*, *Acentria* would, to some extent, also be exposed to secondary compounds leaching from wounded cells.

E. nuttallii crude polar extracts applied to *P. lucens* leaves had the most deleterious effect on *Acentria*. This treatment reduced survival, growth, and feeding (Figs. 3, 4, and 5). The observed mortality with crude polar extract equaled that of live *E. nuttallii* plants (Fig. 3). The increase in headcapsule width of larvae grown with *E. nuttallii* extract was less than 50% of that of *P. lucens* fed larvae and corresponds to the values observed in the assays with fresh plants (Fig. 4). This corroborates our hypothesis that the negative impact of *E. nuttallii* on *Acentria* is mainly due to chemical defenses. Our results indicate that flavonoids can act as feeding deterrents in *E. nuttallii*, but they do not affect survival (Fig. 3). Corresponding to our results, Beninger and Abou-Zaid (1997) observed a high mortality of *Lymantria dispar* when fed crude extract of jack pine, but not when fed jack pine flavonoids alone. Elliger et al. (1980) found several flavonoids that inhibited growth of *Heliothis zea*, but none of them affected survival. Thus, there must be additional unknown, metabolites present in *E. nuttallii* crude polar extracts that exhibit toxicity or act at least additively or synergistically to flavonoids. Our approach opens the possibility of identifying additional relevant signals in a bioassay guided structure elucidation.

The deleterious effect of *E. nuttallii* extracts on *Acentria* confirms earlier suggestions that *Elodea* spp. might be defended against herbivores (Newman 1991; Kornijow et al. 1995). The natural concentrations of the luteolin-derivative in *E. nuttallii* (0.02–1.2% of plant dry mass, see above) and the other two flavonoids (at least 0.014% for chrysoeriol glucuronide and 0.02% for apigenin glucuronide; Mues 1983) are comparable to the content of structurally related allelochemicals found in other plant species (Beninger and Abou-Zaid 1997). Even though the enrichment of *P. lucens* with *E. nuttallii* extracts yielded concentrations only at the lower range found in field *E. nuttallii*, our observed effects suggest that chemical defense should be relevant under natural conditions. All three flavonoids are also present in *E. canadensis* (Mues 1983; Erhard et al. unpublished results.), which is similarly avoided by the moth (Gross et al. 2001), but they do not occur in *P. lucens* or *M. spicatum* (data not shown), which suffer from severe feeding damage by *Acentria* in the field (Gross et al. 2002). Both the effect of *Elodea* secondary allelochemicals as feeding deterrents and inhibitors of survival and growth might reduce the risk of herbivory on *Elodea*.

Our results show that potential allelochemicals are present in *Elodea* leaves as well as coated *Potamogeton* leaves in low but ecologically relevant concentrations, comparable to concentrations effective in terrestrial systems (Beninger and Abou-Zaid 1997). We thus

assume that the chemical defense in *E. nuttallii* is a powerful trait to protect plants against herbivores and might further strengthen the invasiveness of this species.

Acknowledgments We acknowledge the help of Julia Hoffmann, Nadine Womann, and Manuela Mayer during the feeding assays. Vera Bauke is acknowledged for help during the extraction and purification of flavonoids. Heather Fyson improved the English, and two anonymous reviewers made valuable comments on the manuscript. This study was supported by the German Science Foundation (DFG, project A2 in SFB454 to EMG) and the University of Konstanz (AFF).

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Materials and Methods

The amber piece containing the beetle is rectangular, measuring 7 mm long by 4 mm wide by 2 mm deep. The fossil was collected from lignitic seams in sandstone–limestone deposits in the Hukawng Valley in Myanmar. The mine site was located on the slope of the Noije Bum hill about a mile (1.5 km) SSW of the old Khanjamaw mine site and southwest of Maingkwan (26°2′N, 96°36′E). This site is newly mined (D. Cruickshank, personal communication, December 20, 2006) and has been designated as the "Noije Bum 2001 Summit Site." Paleontological findings from this site assigned its age to the Upper Albian (100 Ma BP) of the Early Cretaceous (Cruickshank and Ko 2003). On the basis of spectroscopic and anatomical evidence, the source of Burmese amber from this site was determined to be a member of the Araucariaceae (Poinar et al. 2007). The specimen was deposited in the amber collection of Ron Buckley under accession no. ABS66.

Results

The fossil is approximately 4.5 mm in length, well preserved, complete, and in a clear piece of amber. The beetle is a member of the family Cantharidae and will be described separately. Cuticular vesicles with associated gland reservoirs were extruded from the lateral membrane on each side of the first six abdominal segments (Figs. 1a,b and 2a,c). Secretions were released from the first three vesicles on both sides of the abdomen. By far, the largest deposit (1.4 mm in length) had been released from the extruded vesicle on the right side of the first abdominal segment at the point of contact with a filiform, setaceous, multisegmented antenna belonging to an unknown, alien insect. In this deposit are a number of spherical to subspherical crystal-like bodies ranging from 22 to 34 μm in greatest diameter (Fig. 1c).

Discussion

The extruded vesicles on the fossil soldier beetle are similar in size and shape to those described by Sulc (1949) on a European species of *Cantharis*. Soldier beetles (Coleoptera: Cantharidae) are small- to moderate-sized insects with weakly sclerotized or leathery wing covers. The relatively short-lived adult beetle is active during the day in search of food, which is mainly small insects and pollen from a variety of plants (Dillon and Dillon 1961; Crowson 1981; Daly et al. 1998). Although cantharids are widely distributed today, fossils of this group are unusual, and until the present report the oldest described fossil soldier beetles were from Baltic amber (40 Ma; Spahr 1981; Carpenter 1992). The present discovery more than doubles the previous fossil record.

The structure of the antenna of the potential predator is consistent with those of members of the orders Orthoptera and Blattaria, and the antenna passes through the entire amber piece along the right side of the beetle (Figs. 1a and 2a,b). Members of the above groups that existed at that period and had this type of antennal structure were katydids, crickets, elcanids, and cockroaches, all of which have been reported in Burmese amber (Poinar et al. 2005). This insect probably extended its antennae to investigate the soldier beetle as a future meal. As soon as the beetle detected the intruder's presence, it presumably extruded its abdominal vesicles and emitted defensive secretions (Fig. 3).

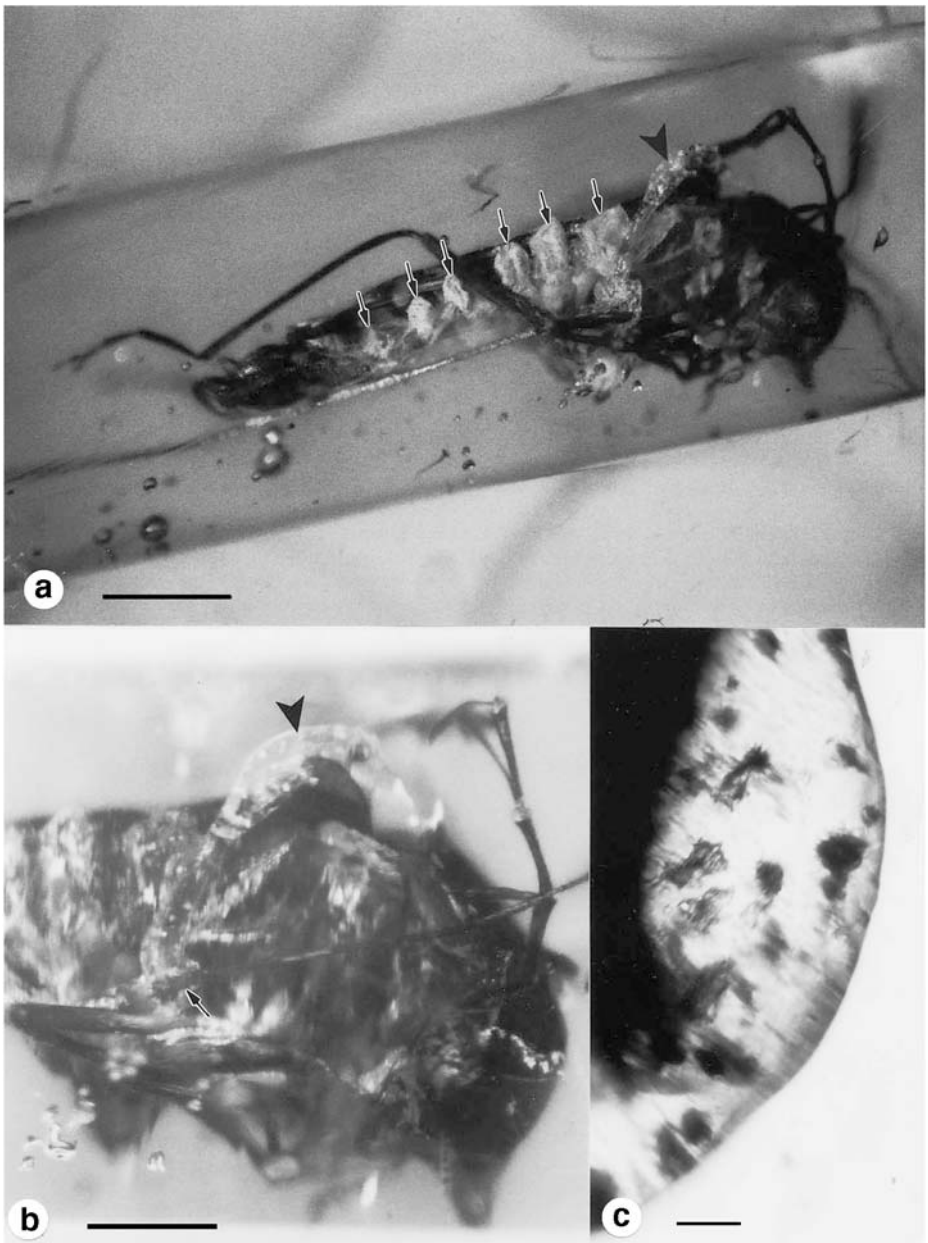


Fig. 1 **a** Soldier beetle in Burmese amber showing chemical defense behavior. Lateral view showing extruded vesicles (*arrows*) on abdominal segments 1 to 6. *Bar*=800 μm. **b** Soldier beetle in Burmese amber showing chemical defense behavior. Detail of anterior region of beetle showing deposit from extruded vesicle on the first abdominal segment covering a portion of the foreign antenna (*arrow*) and the remainder of the excretion extending over the beetle's thorax (*arrowhead*). *Bar*=370 μm. **c** Soldier beetle in Burmese amber showing chemical defense behavior. Crystals and crystal aggregations in the extruded deposit. *Bar*=45 μm

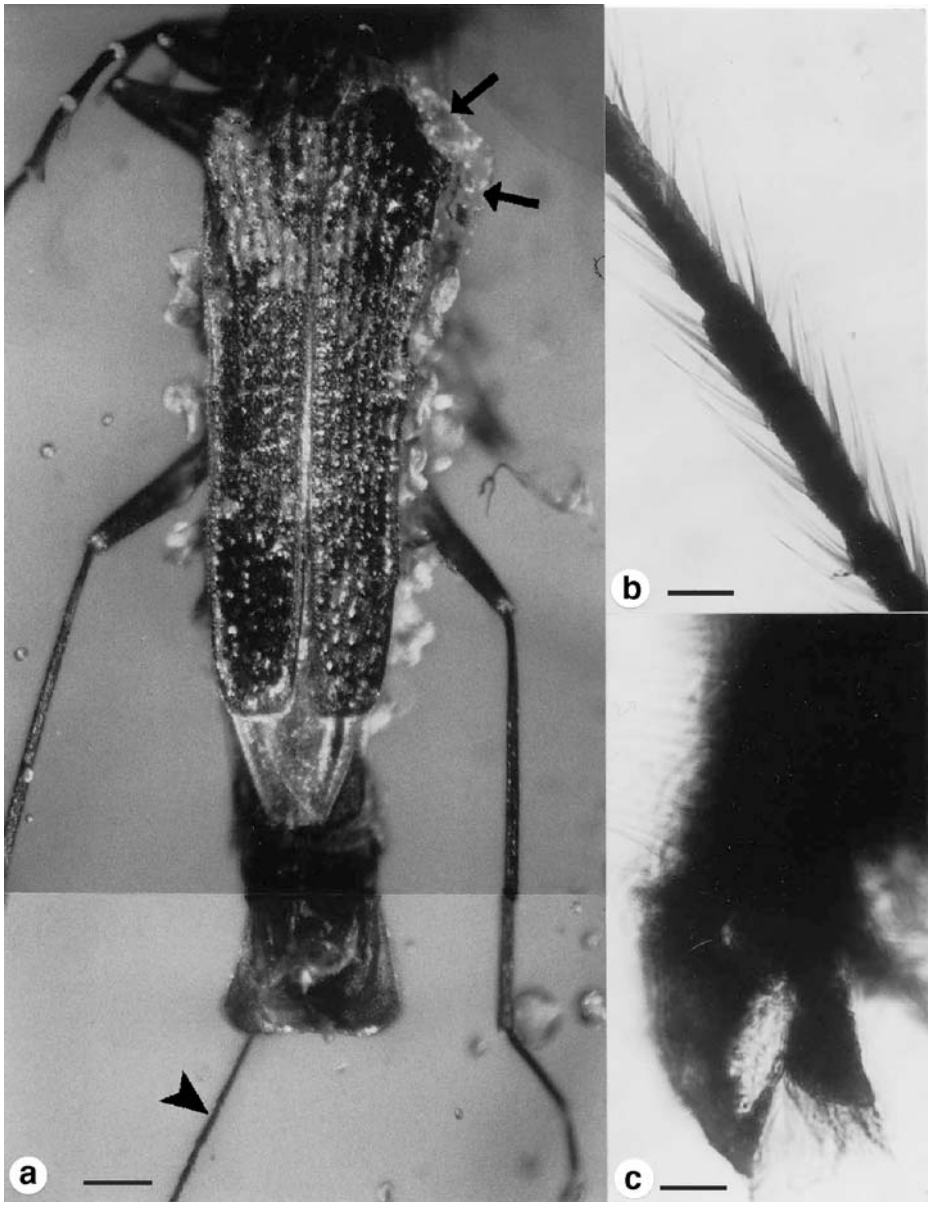


Fig. 2 **a** Soldier beetle in Burmese amber. Dorsal view showing large deposit (*arrows*) from extruded vesicle on the right side of the body. *Bar*=176 μm. **b** Soldier beetle in Burmese amber. Detail of a portion of the alien antenna. *Bar*=38 μm. **c** Soldier beetle in Burmese amber. Extruded vesicle from the fifth abdominal segment on left side of body. *Bar*=21 μm

The vesicle nearest the area of direct molestation (where the foreign antenna made physical contact with the beetle) deposited the largest amount of secretion. Many insects only discharge defensive droplets adjacent to the area of tactile molestation (Blum 1981), thus conserving secretions. Some of the secretion was deposited on the orthopteroid antenna, whereas the remainder was ejected over the insect's right shoulder (Fig. 1b). A

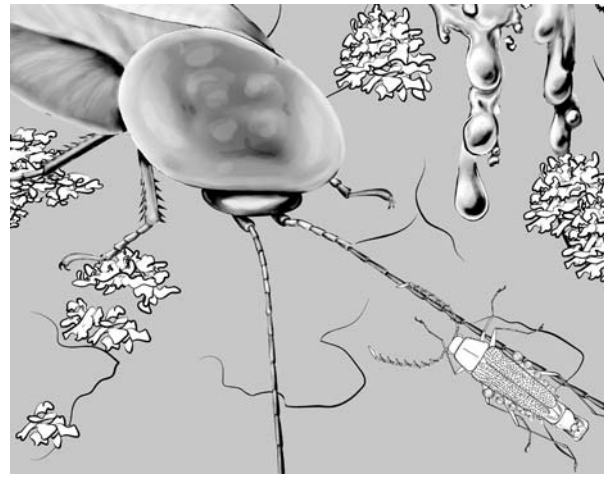


Fig. 3 Proposed scenario on the bark of the resin-producing tree just before the soldier beetle was entombed. In the *upper left* is a blattid with one antenna extended along the right side of the beetle. The beetle is responding by everting six pairs of cuticular vesicles with associated gland reservoirs. A drop of resin in the *upper right* is falling on the beetle. Patches of lichens and moss are growing on the surface of the bark. We portrayed a cockroach as the bearer of the alien antenna because they occur on bark, are omnivorous, and are known from Burmese amber

moment after the beetle released its secretions, it was covered by a flow of resin. Whether the entire predator was also captured is unknown; however, its remaining antenna provides evidence of its presence. This scenario would explain why the preserved beetle was likely employing a defense response, why most of the extruded secretion came from only one everted vesicle, and how some of the secretion ended up on the perpetrator's antenna.

Chemical defense mechanisms have evolved at least 30 times in Coleoptera, including in all members of the Adephaga and in representatives of at least 20 families of Polyphaga (Blum 1981; Dettner 1987). Aside from chemicals produced in specialized glands, distasteful, emetic, or caustic compounds in insect hemolymph are also employed in defense responses (Blum 1981; Dettner 1987). Toxic hemolymph can be released from openings positioned between segments (reflexive bleeding) or via cuticular vesicles extruded from the abdomen and thorax.

Some examples of chemical defense mechanisms involving cuticular vesicles in various families of beetles are listed here. Secretions expelled from extrusive sacs located on the abdomen and thorax of tenebrionid beetles contain benzoquinone, toluquinone, ethylquinone, phenols, aldehydes, and ketones (Tschinkel 1969, 1975a, b). Adult and larval leaf beetles (Chrysomelidae) respond to tactile stimulation by a potential predator by releasing hemolymph with defense compounds (Hilker and Schulz 1994) through cuticular openings positioned along the edges of the thorax and first seven abdominal segments (Pasteels et al. 1988). In disturbed galaerucine (Chrysomelidae) larvae, hemolymph is expelled from extruded dorsolateral vesicles (Bünnige and Hilker 2005). This sticky deposit, which may also contain glandular secretions, can entangle the mouthparts of invertebrate antagonists. After expelling the chemical defenses, these vesicles are retracted back into the body.

Malachiinae (Melyridae) beetles also contain extrusive vesicles located on the abdominal and thoracic segments. Adults of the New Guinea genus *Choresine* contain secondary bioactive compounds called batrachotoxins in their hemolymph, which are apparently expelled through their protruded vesicles located on abdominal segments 1 to 6 and on the prothorax and metathorax (Dumbacher et al. 2004).

It was not possible to identify the crystals in the droplet released by the fossil soldier beetle because any attempted analysis would probably destroy the specimen, but the crystals likely represent defensive chemicals. Soldier beetles of the genus *Chauliognathus* produce certain types of carboxylic acids as well as triglycerides and glyceride esters from

paired exocrine glands located on the thorax and abdomen (Meinwald et al. 1968; Moore and Brown 1978; Eisner et al. 1981; Brown et al. 1988). Diterpenes have also been recovered from members of the genus *Cantharis* (Durvaux et al. 2007). These products seem to be unique to cantharids and could possibly be acquired by feeding on certain types of plants (Blum 1981).

Today, defensive chemicals play an important survival role in insects, but just when beetles began producing toxic compounds in specialized exocrine glands is unknown. The present discovery shows that members of the family Cantharidae had established a chemical arsenal for defense some 100 Ma ago.

Acknowledgements Thanks are extended to Art Boucot and Roberta Poinar for comments on earlier drafts of this study.

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Introduction

Carnivorous arthropods are able to use herbivore-induced plant volatiles as cues for their foraging behaviors in many systems that consist of plants, herbivores, and natural enemies (e.g., Dicke 1994, 1999; Turlings et al. 1995; Takabayashi and Dicke 1996; Dicke and Vet 1999). Herbivorous prey are usually under strong selection not to reveal themselves and are difficult for predators to detect. In contrast, information emitted by plants is easier for a carnivorous arthropod to detect because plants have a large biomass compared to that of its herbivores (Dicke and Sabelis 1989; Vet and Dicke 1992; van Loon et al. 2000). The composition of herbivore-induced plant volatiles differs across plant species, cultivars, and herbivore species and is affected by abiotic factors as well (Loughrin et al. 1994; Takabayashi et al. 1994; Turlings et al. 1995; De Moraes et al. 1998; Gouinguene and Turlings 2002; Schmelz et al. 2003). Therefore, carnivores are confronted with variable information about the presence of prey, especially when both they and their herbivorous prey are polyphagous.

The predatory mite *Neoseiulus womersleyi* (Shicha) (Acari: Phytoseiidae), which is a specialist predator of tetranychid mites, is attracted to volatiles emitted from plants infested with prey (Maeda et al. 1999). Because tetranychids are polyphagous herbivores that induce various volatile compositions in different host plants (Van Den Boom et al. 2004), *N. womersleyi* is likely confronted with various chemical cues about the presence of prey on plants. The olfactory response of *N. womersleyi* shows genetic variation and is influenced by an individual's foraging experience with different plant–prey complexes (e.g., Maeda et al. 2001, 2006; Maeda 2006). For example, *N. womersleyi* individuals were attracted to the volatiles from infested plants (tea/kidney bean) that they had previously experienced, but they were not attracted to volatiles of plants (kidney bean/tea) that they had no experience with (Maeda et al. 2006). Because the composition of volatiles differs between tea and kidney beans (Maeda et al. 2006), *N. womersleyi* may have responded to the plant-specific composition of multiple volatiles or to a single specific volatile component.

In the present study, we identified those components in volatile blends emitted from different prey-infested plants (tea and kidney bean) that are attractive to *N. womersleyi* by testing the attractiveness of mixtures of synthetic compounds. The bioassays were conducted after rearing the predatory mites on each plant species.

Methods and Materials

Plants Tea plants (*Camellia sinensis* 'Yabukita') were grown in a plantation at the National Institute of Agrobiological Sciences (Tsukuba, Japan) for 3 yr. During this time, they were fertilized with ammonium sulfate (2.5 g per plant) every 2 mo. We found no *Tetranychus kanzawai* on the tea plants during this period. Because the densities of other herbivores were below detectable levels, no pesticide was sprayed.

Kidney bean plants (*Phaseolus vulgaris* 'Nagauzura') were grown in plastic pots filled with vermiculite and given ample water (no fertilizer) in a climate-controlled room (19±1°C, 50–70% r.h., 16L:8D at approximately 4,000 lx). When the two primary leaves had fully unfolded (about 2–3 wk after germination), the plants were used in the experiments.

Herbivorous Mites The *T. kanzawai* used in this study had been maintained on tea leaves in the laboratory for more than 1 yr (25±3°C, 60–80% r.h., 16L:8D). Current-year branches of tea plants with five to seven leaves were offered to the *T. kanzawai* every 2 wk.

The *Tetranychus urticae* used in this study were obtained from a laboratory culture that had been maintained on kidney bean plants for more than 2 yr (25±3°C, 60–80% r.h., 16L:8D). About 70 kidney bean plants with two fully unfolded primary leaves were offered to the *T. urticae* culture every week.

Predatory Mites We collected *N. womersleyi* from tea plants infested with *T. kanzawai* at Kanaya, Shizuoka prefecture, Japan (34°48′20″N, 138°08′10″E, 200 m a.s.l.) in June 2003. To maintain the predator population with *T. kanzawai*-infested tea leaves, we used an acrylic resin plate (12.5×17×0.2 cm) with 24 holes (6 lines×4 rows, 2.5-cm intervals, 3-mm diameter). The plate was placed on a water-soaked floral oasis (12.5×17×3.5 cm; Nisso Green, Tokyo, Japan) in a plastic box (24×33 cm, 10-cm depth) whose lid had a rectangular aperture (17×26 cm) covered with nylon gauze (0.2-mm mesh). Petioles of tea leaves infested with *T. kanzawai* (10–20 adult females per leaf, as well as other stages) were inserted into the oasis through the holes in the plate. We added 12 new *T. kanzawai*-infested leaves to the culture three times per week. Under these conditions, 40–60 adult female predatory mites were maintained per generation.

To maintain the predators with *T. urticae*-infested kidney bean leaves, we used an acrylic resin plate (12.5×17×0.2 cm) with no holes. The plate was placed on a water-soaked floral oasis in the plastic box described above. Five kidney bean leaves infested with *T. urticae* (30–50 adult females per leaf, plus other stages) were offered to the predatory mites three times per week. Under these conditions, 40–60 adult female predatory mites were maintained per generation.

Olfactometer Experiments The olfactory responses of the *N. womersleyi* were tested in a Y-tube olfactometer (Takabayashi and Dicke 1992) with a 10-cm arm length, 12-cm stem length, and 4-cm internal diameter. Air (2 l/min) cleaned by granular activated charcoal was passed through both arms of the Y-tube olfactometer. We used excised leaves or volatile extracts as odor sources. In experiments with plants, we confined sample plants in a 1-l glass bottle and connected the bottle directly adjacent to the Y-tube. As a control, we used an empty glass bottle. In experiments with extracts or synthetic compounds, 10 μl of hexane-diluted odor sample (see below) were applied to a piece of filter paper (8 cm^2). After evaporation of the solvent, the filter paper was placed in one arm of the Y-tube olfactometer. Another filter paper with 10 μl hexane was placed at the same position in the other arm as a control. Filter papers were changed after five mites had been tested. We confirmed that the olfactory response of *N. womersleyi* was not affected by the sequence (i.e., first to fifth) of tested individuals based on results of preliminary experiments (logistic regression analysis, $P=0.649$, $N=50$).

Fifty *N. womersleyi* individuals (25 individuals/day) were tested for each odor source during the daytime in a climate-controlled room (25±2°C, 50–70% r.h., approximately 1,500 lx). Gravid females of *N. womersleyi* were placed individually in 0.5-ml plastic tubes without prey and water at least 200 min before each bioassay, and each was then individually introduced at the starting point (the downwind end) of a Y-shaped steel wire in the center of the olfactometer. The behavior of each was observed until the mite walked across one of the finish lines established on the upwind ends of the Y-shaped wire. A preliminary examination showed that the mean time to reach the finish line was 102±25 sec (mean±SE, $N=50$).

To account for any unexpected bias caused by the experimental setup, such as the shapes of the Y-shaped glass tube and the steel wire, after every five mites, the arms of the olfactometer were reversed between the sample and control odor sources and the steel wire was replaced. When no odor source was present in the odor-source chamber, predatory

mites were equally attracted to the airflow from both arms of the olfactometer (48% vs. 52%, two-tailed binomial test, $P>0.05$, $N=50$).

Odor Sources to Test the N. womersleyi Reared on T. kanzawai-infested Tea Leaves To investigate the essential components in volatiles of *T. kanzawai*-infested tea leaves that attract *N. womersleyi*, eight odor sources were tested.

(1) *T. kanzawai*-infested tea leaves: To prepare the *T. kanzawai*-infested tea leaves, we maintained five branches of tea plants (each with three leaves) on a water-soaked floral oasis. We introduced five adult females of *T. kanzawai* onto each leaf. This prey density per leaf is commonly observed in tea plantations (e.g., Kawai 1997). After 10 d of the first infestation (25±1°C, 70±5% r.h., 16L:8D at approximately 10,000 lx), the infested tea leaves were used for bioassays. This procedure was the same one used by Maeda et al. (2006). Although Baldwin et al. (2002) reported that excised plant tissues released far more volatiles from both infested and uninfested tissues than was the case for comparable tissues of intact (rooted) plants (corn and lima bean), our preliminary tests confirmed that excised tea branches and rooted plants released similar amounts of volatiles after being infested with *T. kanzawai*.

(2) Uninfested tea leaves: We maintained five branches of tea plants (each with three leaves) on a water-soaked floral oasis. After 10 d of incubation (25±1°C, 70±5% r.h., 16L:8D at approximately 10,000 lx), the branches were used for bioassays.

(3) Extraction of collected volatiles of *T. kanzawai*-infested tea leaves: We confined 5 *T. kanzawai*-infested branches prepared by using the same procedure mentioned above (15 leaves total) in a 1-l glass bottle. We then collected the headspace volatiles for 5 hr with an ORBO-91T adsorbent tube (SUPELCO, Bellefonte, PA, USA) at a flow rate of 100 ml/min of clean air, purified through activated charcoal. The adsorbent tube was washed with 2.0 ml of dichloromethane (environmental analysis grade, Wako Pure Chemical Industries, Osaka, Japan) to extract the volatiles. The sample containing the volatiles was concentrated through evaporation with a rotary evaporator and then diluted with hexane (96% pure, Wako Pure Chemical Industries) to 75 μl. The emission of volatiles by plants is affected by several factors, including prey density, light conditions, and the developmental stage; in this study, however, the condition of the plants did not change during the volatile-collecting period (5 hr). We confirmed that the amount of volatiles did not change over the course of the collection period by comparing the amounts of volatiles emitted during the first, third, and fifth hours. For example, the amounts of (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E*,*E*)-α-farnesene (peak area/hr/leaf) did not differ among each period [(35±7, 32±4, and 35±6), (283±43, 263±19, and 294±30), and (925±121, 908±151, and 904±180), respectively, $N=3$]. Therefore, 10 μl of the end product applied on a piece of filter paper included 10 leaf-hr equivalents of volatiles.

(4) Extraction of collected volatiles emitted from uninfested tea leaves: The volatiles of 5 uninfested tea branches (15 leaves total) were collected and concentrated by using the same procedure described above, and 10 μl of the end product included 10 leaf-hr equivalents of volatiles.

(5) Synthetic mixture of volatile compounds of the *T. kanzawai*-induced tea leaf volatiles: Because *T. kanzawai*-infested tea leaves emit significantly larger amounts of (*E*)-β-ocimene, DMNT, and (*E*,*E*)-α-farnesene than do uninfested plants (Maeda et al. 2006), we used these compounds as the herbivore-induced plant volatiles of tea leaves. The quantities of these compounds were tested with an internal standard

method that used dodecane (99% pure, Wako Pure Chemical Industries). An infested tea leaf emitted 19.5 ng of (*E*)-β-ocimene, 98.4 ng of DMNT, and 411.4 ng of (*E*,*E*)-α-farnesene per hr. (*E*)-β-Ocimene (93% pure) and DMNT (95% pure) were synthesized in our laboratory and were identified by ^{1}H (600 MHz) and ^{13}C (125 MHz) NMR analysis (Bruker AVANCE-600, Bruker, Billerica, MA, USA; Matsushita and Negishi 1982; Maurer et al. 1986). (*E*,*E*)-α-Farnesene (>99% pure) was purified from apple pericarp and identified by means of NMR analysis (Pinheiro et al. 1984). We dissolved the three compounds as 10 leaf-hr equivalents in 10 μl of hexane [200 ng of (*E*)-β-ocimene, 1,000 ng of DMNT, 4,000 ng of (*E*,*E*)-α-farnesene].

To test whether each compound is essential for attraction, two of the three compounds were dissolved into hexane (10 leaf-hr equivalents per 10 μl) and were used as attractant as follows:

(6) synthetic mixture without (*E*)-β-ocimene [DMNT and (*E*,*E*)-α-farnesene];
(7) synthetic mixture without DMNT [(*E*)-β-ocimene and (*E*,*E*)-α-farnesene];
(8) synthetic mixture without (*E*,*E*)-α-farnesene [(*E*)-β-ocimene and DMNT].

The eight types of samples were tested by two-choice experiments with hexane (or air) as a control odor source. In addition, we performed a two-choice experiment to test whether the mixture of the three synthetic compounds [(*E*)-β-ocimene, DMNT, and (*E*,*E*)-α-farnesene] and the volatiles collected from *T. kanzawai*-infested tea leaves attracted the predatory mites.

Odor Sources to Test the N. womersleyi Reared on T. urticae-infested Kidney Bean Plants To investigate the essential components in volatiles of *T. urticae*-infested kidney bean plants that attract *N. womersleyi*, nine odor sources were tested.

(1) *T. urticae*-infested kidney bean plants: To prepare the *T. urticae*-infested kidney bean plants, 30 adult females of *T. urticae* were introduced onto each leaf of 5 intact bean plants with two primary leaves (i.e., a total of 300 mites on 5 plants) and reared in a climate-controlled incubator for 10 d (25±1°C, 70±5% r.h., 16L:8D at approximately 10,000 lx). This procedure was similar to that used by Maeda et al. (2006), except for the temperature and infestation period. The plants were reared without excision.
(2) Intact kidney bean plants: Five kidney bean plants, each with two primary leaves, were maintained in a climate-controlled incubator (25±1°C, 70±5% r.h., 16L:8D at approximately 10,000 lx) for 10 d.
(3) Extraction of collected volatiles of *T. urticae*-infested kidney bean plants: We collected the headspace volatiles of *T. urticae*-infested kidney bean plants prepared by using the same method described above. The extract of the collected volatiles was diluted with hexane so that 10 μl of the end product applied on a piece of filter paper included 10 leaf-hr equivalents of volatiles.
(4) Extraction of collected volatiles emitted from intact kidney bean plants: The volatiles of intact plants were collected and concentrated so that 10 μl of the end product included 10 leaf-hr equivalents of volatiles.
(5) Synthetic mixture of volatile compounds of the *T. urticae*-induced kidney bean plant volatiles: Maeda and Liu (2006) showed that *T. urticae*-infested kidney bean plants emit significantly larger amounts of DMNT, methyl salicylate (MeSA), and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) than do intact plants. They used a TENAX adsorbent tube. In this study, analysis with an ORBO-91T adsorbent tube

showed that significantly greater amounts of β-caryophyllene also occurred in the infested plant volatiles compared to those of uninfested plants. β-Caryophyllene was found in the headspace of *T. urticae*-infested kidney bean plants by using SPME fiber (Maeda and Takabayashi 2001). Therefore, in this study, we used these compounds (DMNT, MeSA, β-caryophyllene, and TMTT) as the herbivore-induced volatiles of kidney bean plants. The quantities of these compounds were tested with an internal standard method with dodecane. An infested leaf emitted 203.3 ng of DMNT, 14.8 ng of MeSA, 2.7 ng of β-caryophyllene, and 23.4 ng of TMTT. TMTT (92.3% pure) was kindly provided by Dr. Kugimiya of the Center for Ecological Research, Kyoto University. MeSA (98% pure) and β-caryophyllene (89% pure) were obtained from Wako Pure Chemical Industries. We dissolved the four compounds as 10 leaf-hr equivalents in 10 μl of hexane (2,000 ng of DMNT, 150 ng of MeSA, 50 ng of β-caryophyllene, and 250 ng of TMTT).

To investigate the requirement of each compound for attraction, three of the four compounds were used in various mixtures as follows:

(6) synthetic mixture without DMNT (MeSA, β-caryophyllene, and TMTT);
(7) synthetic mixture without MeSA (DMNT, β-caryophyllene, and TMTT);
(8) synthetic mixture without β-caryophyllene (DMNT, MeSA, and TMTT);
(9) synthetic mixture without TMTT (DMNT, MeSA, and β-caryophyllene).

The nine types of samples were tested by two-choice experiments with hexane (or air) as a control odor source. In addition, we used two-choice experiments to test whether the mixture of the four synthetic compounds (DMNT, MeSA, β-caryophyllene, and TMTT) and the volatiles collected from *T. urticae*-infested kidney bean plants attracted the predatory mites.

Statistical Analysis The data from each bioassay experiment were analyzed by using a two-tailed binomial test. The null hypothesis was that predators exhibit a 1:1 distribution over the two odor sources. To test the requirement of each synthetic compound, the degree of attractiveness was compared between a mixture containing all the synthetic compounds *versus* a mixture without one of the compounds (Fisher's exact probability tests).

Results

Response to the Volatile Compounds Emitted from Tea Leaves Tea leaves infested with *T. kanzawai* attracted more than 50% of the predatory mites that had been reared with the *T. kanzawai*-infested tea leaves (74%; two-tailed binomial test, $P<0.001$; Fig. 1), whereas uninfested tea leaves did not attract these predatory mites (two-tailed binomial test, $P>0.05$; Fig. 1). Likewise, the extract of *T. kanzawai*-infested tea leaves attracted more predatory mites (74%; two-tailed binomial test, $P<0.001$; Fig. 1), whereas the extract of uninfested tea leaves did not attract these mites. More than 50% of the predators were attracted to the mixture of (*E*,*E*)-α-farnesene, (*E*)-β-ocimene, and DMNT (74%; two-tailed binomial test, $P<0.001$). In the absence of even one of the *T. kanzawai*-induced volatiles, the mites did not show any preference for the volatiles (two-tailed binomial test, $P>0.05$; Fig. 1). When either (*E*,*E*)-α-farnesene or DMNT was not included in the odor source, the attractiveness of the two-compound mixture was significantly less than that of the three-compound mixture (Fisher's exact probability test, $P=0.004$ and 0.023, respectively). When the extract

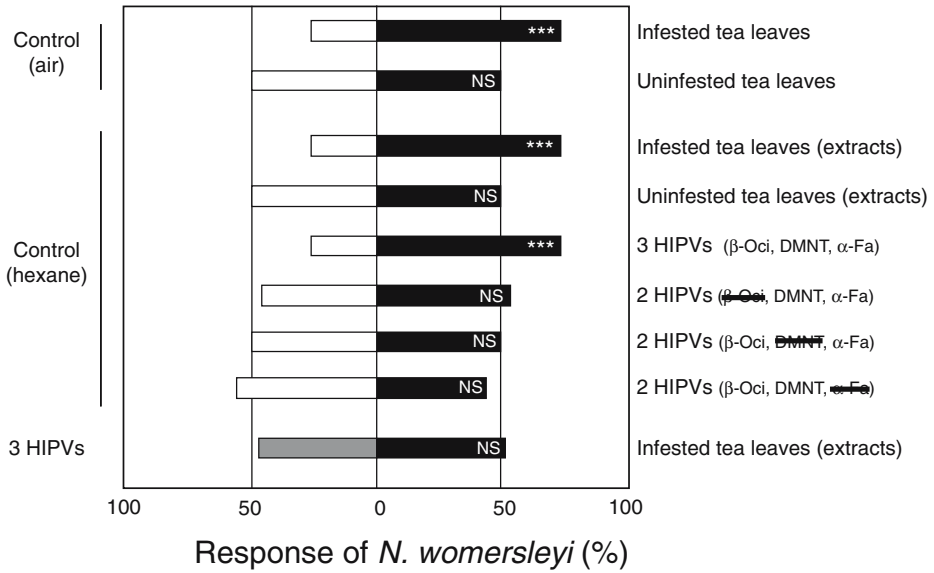


Fig. 1 Responses (%) of *N. womersleyi* to volatiles emitted from *T. kanzawai*-infested tea leaves, uninfested tea leaves, collected volatiles (extracts) from *T. kanzawai*-infested tea leaves, extracts of uninfested leaves, and various combinations of synthetic compounds. The three herbivore-induced plant volatiles (HIPVs) tested were β-ocimene (*β-Oci*), (*E*)-4,8-dimethyl-1,3,7-nonatriene (*DMNT*), and (*E*,*E*)-α-farnesene (*α-Fa*). The upper eight bars show the results of a two-choice test between the sample odors (*black bars*) vs. control odor sources (air or hexane; *white bars*). The bottom bar shows the results of a two-choice test between the collected volatiles from the infested tea leaves (*black bar*) and the mixture of the three synthetic compounds (*gray bar*). ***$P<0.001$

of *T. kanzawai*-infested tea leaves and the synthetic mixture were compared directly, the predatory mites did not show any preference (two-tailed binomial test, $P>0.05$; Fig. 2).

Response to the Volatile Compounds Emitted from Kidney Bean Plants Kidney bean leaves infested with *T. urticae* attracted more than 50% of the predatory mites that had been reared with the *T. urticae*-infested kidney bean leaves (82.0%; two-tailed binomial test, $P<0.001$; Fig. 2), whereas intact plants did not attract the predatory mites. Likewise, *N. womersleyi* showed a significant preference for the extract of the *T. urticae*-infested kidney bean plants (72.0%; two-tailed binomial test, $P<0.01$; Fig. 2), whereas they were not attracted to the extract of the intact kidney bean plants. The mixture of the four *T. urticae*-induced volatile compounds (DMNT, MeSA, β-caryophyllene, and TMTT) attracted significantly more than 50% of these predatory mites (78.0%; two-tailed binomial test, $P<0.001$), and the attractiveness of this odor source was not significantly different from that of the *T. urticae*-infested kidney bean plants or that of the extract of the infested kidney bean plants. However, odor sources lacking any of the volatiles resulted in no preference for the mixture (two-tailed binomial test, $P>0.05$). When DMNT, MeSA or TMTT was excluded from the odor source, the attractiveness of the three-compound mixture was significantly lower than that of the four-compound mixture (Fisher's exact probability test, $P=0.020$, 0.020, and 0.033, respectively). There was no significant preference in the two-choice experiment testing the extract of infested plants and the synthetic mixture (two-tailed binomial test, $P>0.05$; Fig. 2).

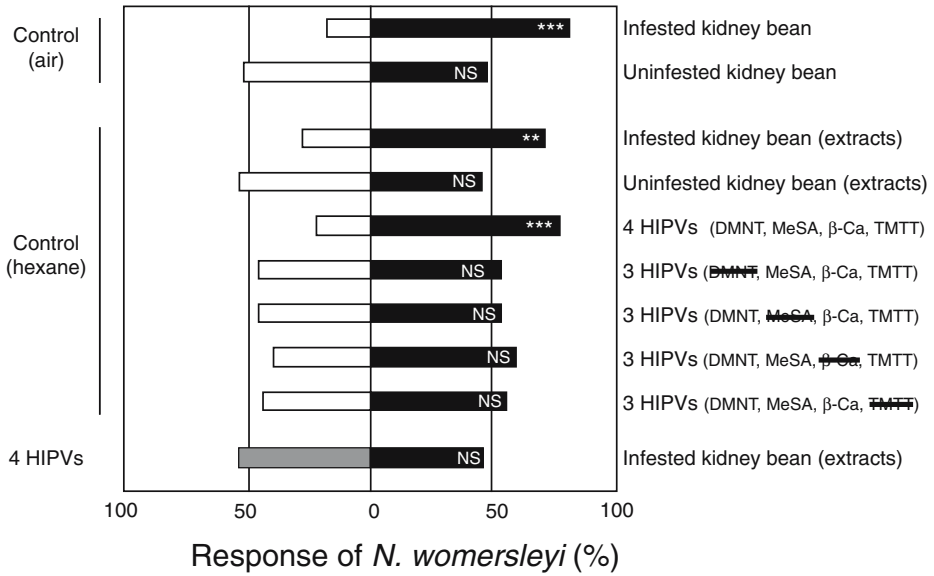


Fig. 2 Responses of *N. womersleyi* to volatiles emitted from *T. urticae*-infested kidney bean plants, intact kidney bean plants, collected volatiles (extracts) from *T. urticae*-infested kidney bean plants, extracts of intact plants, and various combinations of synthetic compounds. The four herbivore-induced plant volatiles (HIPVs) tested were (*E*)-4,8-dimethyl-1,3,7-nonatriene (*DMNT*), methyl salicylate (*MeSA*), β-caryophyllene (*β-Ca*), and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (*TMTT*). The upper nine bars show the results of a two-choice test between the sample odors (*black bars*) vs. control odor sources (air or hexane; *white bars*). The bottom bar shows the results of a two-choice test between the collected volatiles from the infested kidney bean plants (*black bar*) and the mixture of the four synthetic compounds (*gray bar*). ***$P<0.001$; **$P<0.01$

Discussion

The mixture of three synthetic compounds [(*E*)-β-ocimene+DMNT+(*E*,*E*)-α-farnesene, a mimic of *T. kanzawai*-induced tea leaves volatiles] was attractive to *N. womersleyi* that had been reared on tea leaves, but mixtures of any two of these compounds were not attractive. Likewise, the mixture of DMNT, MeSA, β-caryophyllene, and TMTT (a mimic of *T. urticae*-induced kidney bean plant volatiles) was attractive to *N. womersleyi* that had been reared on kidney bean plants, but mixtures of any three of these compounds were significantly less attractive. Therefore, these compounds are the essential attractants among the *T. kanzawai*-induced tea leaves volatiles and *T. urticae*-induced kidney bean plant volatiles, respectively.

In this study, we did not test the attractiveness of a single compound, although Maeda et al. (2006) showed that *N. womersleyi* was not attracted to MeSA alone. In addition, the results of the two- or three-compound mixtures suggest that no single compound tested in this study would attract *N. womersleyi*. Although the effects of other compounds not tested in this study remain unclear, the equal attractiveness of the synthetic mixtures and an equivalent blend of herbivore-infested plant volatiles suggest that the effect of other compounds would be smaller than the three and four tested compounds in the respective plant systems. In the present study, we focused on the essential components of the herbivore-induced plant volatiles that attract the predatory mite. Thus, the synthetic volatiles were tested at a dose equivalent to that produced by the mite-infested plants.

However, as dose-response tests would give us important information, the dose-response tests should be conducted in the future.

Several studies have identified carnivore-attracting compounds from herbivore-induced volatile blends by testing their attractiveness as a single synthetic compound or in a mixture of synthetic compounds (e.g., Whitman and Eller 1992; Scutareanu et al. 1997; Du et al. 1998; Turlings and Fritzsche 1999; Birkett et al. 2003). Only three studies, however, have identified the essential attractants to predatory mites in volatile blends emitted from plants, e.g., lima bean (Dicke et al. 1990a; De Boer et al. 2004; Shimoda et al. 2005). *Phytoseiulus persimilis* (Dicke et al. 1990a) and *Neoseiulus californicus* (Shimoda et al. 2005) showed preferences for several individual prey-induced plant volatile compounds. In another study, *P. persimilis* was attracted to a mixture of several volatiles (De Boer et al. 2004), although that finding could be explained by the attractiveness of a single compound (MeSA). In contrast, our study revealed that *N. womersleyi* showed a preference only for a mixture of several compounds and that the attractiveness could not be explained by a single compound in the mixture.

In nature, plants are usually surrounded by other plants, both conspecifics and heterospecifics. In addition, a single plant may be attacked simultaneously by several herbivore species. Because plant volatile blends are affected by both plant and herbivore species, the predatory mites' environment contains numerous cues. Under such conditions, it would be advantageous for carnivores to be able to distinguish a blend of volatiles emitted from a plant infested by its desired prey (Dicke et al. 2003). De Boer et al. (2004) showed that TMTT alone did not attract *P. persimilis*, but that the presence of TMTT was necessary for *P. persimilis* to distinguish among different volatile blends. Our results also show the importance of several components in a mixture. By using several compounds as cues, *N. womersleyi* should be able to distinguish various blends of volatiles emitted from different plant–prey combinations.

When a specialist predator preys on a monophagous herbivore, the predator may locate its prey by using a specific cue emitted from the plant–prey complex. However, when a predator is a generalist and its prey are polyphagous, the predator must differentiate among and respond to various volatile compounds emitted from the different plant–prey complexes. For instance, the generalist predatory mite *N. californicus* is attracted not only to volatiles from *T. urticae*-infested lima bean leaves but also to volatiles from artificially damaged lima bean leaves, and those from *T. urticae* plus their products such as feces, exuviae, and eggs (Shimoda et al. 2005). In addition, *N. californicus* showed preferences for single synthetic compounds, including (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate, which are commonly released from various plants even when artificially damaged. These findings suggest that this generalist predatory mite is capable of exploiting a variety of volatile infochemicals that originate from its prey, the prey's host plants, and the interaction between the prey and host plants.

Predators such as *N. womersleyi* and *P. persimilis* prefer spider mites of the genus *Tetranychus*. Because many spider mites are polyphagous (Jeppson et al. 1975), *N. womersleyi* and *P. persimilis* must respond to various spider mite–plant complexes. If several plant species emit a common volatile compound when infested with spider mites, then predatory mites could use the common volatile to find their prey. However, such volatiles may be emitted even when nonprey herbivores attack the plants (Dicke 1999). Therefore, the response to such a general volatile would increase the likelihood of the predator making errors and being attracted to inappropriate herbivores. To avoid making such errors, it would be a better strategy to use more specific infochemicals that originate from their prey–plant complex.

The olfactory responses of *N. womersleyi* and *P. persimilis* have been shown to be affected by their experience (Dicke et al. 1990b; Drukker et al. 2000; De Boer et al. 2005;

Maeda et al. 2006), and the flexibility of the olfactory responses appear to be caused by learning (e.g., Drukker et al. 2000; De Boer et al. 2005). Such an ability to learn would enable predatory mites to respond to specific volatile blends. By focusing solely on these learned blends, however, predators would overlook plants that they have not yet experienced but that may be inhabited by suitable prey. Thus, it would be interesting to examine whether it is better for predators to use commonly emitted volatiles or to learn specific volatile blends. In other words, which strategy is more selectively disadvantageous—being attracted to inappropriate plant–prey complexes or overlooking suitable prey? To answer this question, further investigations of various predators' olfactory response to synthetic volatiles and theoretical studies with mathematical models should be conducted.

Acknowledgments We thank Dr. Junji Takabayashi of the Center for Ecological Research, Kyoto University, and Dr. Norihide Hinomoto of the National Institute of Agrobiological Sciences for the critical review of an early draft of this paper. Dr. Soichi Kugimiya from the Center for Ecological Research kindly provided (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. The authors are grateful to the Chemical Analysis Center, University of Tsukuba, for providing the NMR spectra. This work was supported by the Program for Young Researchers with Fixed-Term Appointments, Special Coordination Funds for Promoting Science and Technology, MEXT, Japan.

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Introduction

Interactions among plants, their herbivores, and their pollinators are thought to have led to the diversification of both plants and insects (e.g., Becerra 1997; Farrell and Mitter 1998; Dodd et al. 1999). Historically, studies of plant–herbivore and plant–pollinator interactions have focused either on pollinator attraction to floral traits or herbivore deterrence by plant defenses. However, most plants interact simultaneously with a variety of pollinators and herbivores that could exert reinforcing or conflicting selection pressure on plant traits (Strauss and Whittall 2006; Adler 2007) The study of pair-wise interactions in isolation may not give us an adequate understanding of the costs and benefits of phenotypic traits that mediate interactions with both herbivores and pollinators (Gomez and Zamora 2000; Herrera 2000; Adler et al. 2006).

An increasing number of studies have demonstrated that floral display and rewards may attract antagonists as well as mutualists. For example, wider corollas have made *Polemonium viscosum* flowers more attractive to pollinators but also more vulnerable to ant nectar thieves (Galen and Cuba 2001), and *Ipomopsis aggregata* plants with larger inflorescences attract higher rates of predispersal seed predation (Brody and Mitchell 1997). Hermaphrodites of *Fragaria virginiana* experience more herbivory by the bud-clipping weevil *Anthonomus signatus* compared to female morphs, and this difference is thought to be due to pollen rewards offered by hermaphrodites (Ashman et al. 2004a). Greater nectar rewards in *Datura stramonium* increased oviposition of herbivorous larvae by pollinating hawkmoths (Adler and Bronstein 2004). Therefore, attracting pollinators without also attracting herbivores may be a challenge for flowering plants that require animal pollinators to reproduce.

Scent plays a near-ubiquitous role in plant–insect interactions. Plant volatiles can attract pollinators, provide learning cues for foraging insects, serve as herbivore repellants or attractants, or attract natural enemies of herbivores (De Moraes et al. 1998; Pichersky and Gershenzon 2002; Cunningham et al. 2004; Ashman et al. 2005; Huber et al. 2005). Despite the many ways that scent influences interactions between plants and insects, we know relatively little about how herbivores and pollinators respond to the same scent volatiles. Such information may provide insight into the evolution of the composition, strength, and timing of scent emission. For example, both *Cirsium arvense* and *Nicotiana attenuata* have high floral volatile emission rates during peak reproductive periods that decline after pollination, suggesting a possible cost to emission after pollination is effected (Euler and Baldwin 1996; Theis and Raguso 2005; Theis et al. 2007). However, there are few data to assess the costs and benefits of scent emission in most systems.

Our study addressed the role of floral scent mediating interactions among squash plants, their herbivores, and their pollinators. Most *Cucurbita* species are monoecious, rely on pollinator visitation for reproduction, and are often pollinated by specialist squash bees (Hurd et al. 1974). Several floral volatiles from *Cucurbita* blossoms are attractive to many species of herbivorous cucumber beetles (Andersen and Metcalf 1986; Lampman and Metcalf 1987; Lewis et al. 1990; Metcalf et al. 1995). However, the effect of these volatiles on pollinator attraction is unknown. The goal of this study was to determine whether pollinators and cucumber beetles were attracted to the same floral volatiles to provide a greater understanding of how mutualistic and antagonistic interactions may affect reproductive fitness and selection on *Cucurbita* floral scent.

Methods and Materials

Study System and Site The study was conducted in a butternut squash field located in the town of Dighton, Bristol County, in southeastern Massachusetts, USA (41°49′06.65″ N, 71°07′57.19″ W).

Cucurbita moschata Duchesne (butternut squash, Cucurbitaceae) is an annual broad-leaved vine crop. Plants are monoecious with separate male and female flowers that last for 1 day, opening before sunrise and wilting by early afternoon (McGregor 1976). The scent of male and female *Cucurbita* blossoms attracts *Acalymma vittatum* Fabricius (striped cucumber beetle, Chrysomelidae) (Andersen and Metcalf 1986; Lewis et al. 1990; Metcalf and Lampman 1991; Metcalf et al. 1995; Granero et al. 2004; Ferrari et al. 2006), a specialist herbivore that is responsible for extensive damage to northeastern cucurbit crops (Hoffmann et al. 1996). Leaves and roots of some *Cucurbita* species also produce volatiles that may attract cucumber beetles and other insects (Peterson et al. 1994; Cosse and Baker 1999). However, we focused on floral volatiles because these are most likely to be attractive to pollinators as well as herbivores.

Acalymma vittatum overwinters as adults in diapause that emerge in late spring, feed on cotyledons and new leaves, and then oviposit in early summer. Larvae are specialists on cucurbit roots. The next generation of adults emerges in midsummer and feeds on cucurbit leaves, blossoms, and fruit (Metcalf and Metcalf 1992). Cucumber beetles are often found in cucurbit flowers, where they feed on pollen and floral tissue (Bach 1977; Andersen and Metcalf 1986, 1987; Metcalf and Metcalf 1992).

The squash bee *Peponapis pruinosa* Say (squash bee, Apidae) is one of the most abundant pollinators of squash and pumpkin (Hurd et al. 1974; Shuler et al. 2005). This specialist species collects pollen exclusively from *Cucurbita* blossoms (Hurd et al. 1974). *Apis mellifera* L. (honeybee, Apidae) pollinates cucurbits but does not have a particular affinity (Shuler et al. 2005). Other pollinators that visit *Cucurbita* crops include *Bombus* spp. Latreille (bumblebee, Apidae), various halictid species (*Lasioglossum* spp., *Agapostemon* spp., and Augochlorinae, Halictidae), and *Melissodes bimaculata* Lepeletier (two-spotted miner bee, Apidae) (Shuler et al. 2005).

Treatments Three volatiles, 1,2,4-trimethoxybenzene (T hereafter, Sigma-Aldrich, St. Louis, MO, USA, T68802, 97% pure), indole (I hereafter, Sigma-Aldrich, 13408, 99+% pure), and (*E*)-cinnamaldehyde (C hereafter, Sigma-Aldrich, C80687, 99% pure), are components of *Cucurbita* blossoms that, in combination, were attractive to *A. vittatum* in previous studies and have been used to monitor beetle abundance (Andersen and Metcalf 1986; Lewis et al. 1990; Metcalf et al. 1995). Our purpose was to determine whether these volatiles are also attractive to pollinators to assess whether plants might experience a tradeoff in terms of their ability to attract pollinators while deterring herbivores. The presence and absence of the three volatiles was manipulated in a 2×2×2 factorial design for a total of eight combinations. For each treatment, volatiles were placed separately onto cotton wicks in 20-μl amounts and were applied additively to follow the method of Lewis et al. (1990). Due to its solid state at room temperature, 20 mg of I was dissolved in 20 μl of acetone. Acetone was not used for the other volatiles, which were liquids. Therefore, we used two control treatments, 20 μl of acetone and a dry cotton wick, for a total of nine treatments.

Emission rates vary widely within and among species, and our treatment emission rate is approximately 100-fold higher than what is emitted from various *Cucurbita* on a daily basis (Andersen and Metcalf 1987; Theis, Adler, and Hazzard, personal observation). These rates may be high, but they were selected to mirror those used previously (Lewis et al. 1990). Further, *A. vittatum* attraction had a tightly positive log-linear response to concentrations of both indole and TIC between 0.3 and 30 mg per trap (Lewis et al. 1990). While the slope of the response was higher for TIC than indole, this suggests that beetle response may change in magnitude, but not direction, due to higher concentrations of volatiles. However, similar dose–response curves have not been conducted for *P. pruinosa*. There was 20 μl of volatile

in treatments with a single component (T, I, and C alone), 40 µl of total volatile in treatments with two components (T + I, T + C, I + C), and 60 µl of total volatiles in treatments with all three components (T + I + C). The processing of mixtures of distinct volatiles in the insect brain appears to be close to additive, or even slightly suppressive (Deisig et al. 2006), and so the increase in total volatiles in the combined treatments should not by itself increase attraction.

Trap Design Based on preliminary tests of white, silver, and yellow bowls (Solo Cup, Highland Park, IL, USA) in two sizes (540 ml vs. 4.8 l), we selected yellow (stock no. SPB18), to serve as the trap base because it attracted the most insects (data not shown), which is consistent with previous research using other trap types (Hoffmann et al. 1996). Preliminary trapping indicated that bowl size did not affect insect trapping (data not shown), so the 540-ml bowls were used because they were similar in size to a butternut squash flower. Traps were composed of a bowl with a 20-ml scintillation vial attached to the bottom with silicone-based glue (Permatex, clear RTV silicone adhesive sealant, model 66B, Permatex, Hartford, CT, USA). A 2-ml graduated microcentrifuge tube was placed inside the 20-ml vial. A piece of cotton roll (TIDI Brand, nonsterile, #2-medium, TIDI Products, Neenah, WI, USA) was cut to 3 mm×1 cm and placed into each centrifuge tube. Traps were filled with 520 ml of a soap–water solution (unscented Dawn® soap, Proctor and Gamble, Cincinnati, OH, USA).

Experimental Design Twenty-seven traps were placed in the field on July 26, 2005. Traps were arranged in three blocks, each of which was a row that included one trap in each treatment combination. Traps within blocks were approximately 3 m apart, alternating every other plant, and positioned underneath a broad leaf to protect from rain and mimic flower position. Volatiles were replaced everyday between 1800 and 1900 hours, just before sunset, which allowed us to monitor squash bee pollinators that began to forage before sunrise (Hurd et al. 1974). To avoid bias due to edge or positional effects, traps were moved over one position within the block each day.

Insect Collection and Identification Insects were collected on two separate dates, July 31 and August 4, so that each collection represented multiple days of cumulative insect trapping. Butternut squash plants began to bloom on July 31, reducing the number of pollinators found in traps in the second collection. Insects were cleaned and dried following a protocol from the USDA Bee Biology and Systematics Laboratory (2002). Pollinators were identified to genus using Michener et al. (1994) and to species using Mitchell (1960, 1962).

Data Analysis The number of *P. pruinosa* and *A. vittatum* per trap were used as response variables in separate analyses. Other insects were not caught in sufficient numbers to be analyzed (see "Results" section, "Insect Composition and Abundance" subsection). The two controls (acetone vs. no solvent) were combined into one treatment because there was no difference between them for either insect group ($F_{1,\ 11}<0.1$, $P>0.75$ for *A. vittatum*; no *P. pruinosa* caught in either control). We then determined the effect of T, I, and C as fixed main effects (presence/absence of each), their interactions, block, and collection date on *P. pruinosa* and *A. vittatum* abundance by using separate ANOVAs. Trap was the unit of replication. The number of *A. vittatum* was $\log(x+1)$ transformed to meet assumptions of normality; *P. pruinosa* abundance was normal without transformation. Mean position was used as a covariate for the pollinator analysis but dropped from the beetle analysis because it was not significant. All analyses were performed with SAS v. 9.1.

We expect that synergistic effects of individual compounds will be reflected by significant interaction terms in our ANOVA analysis. However, synergism was defined by Metcalf et al. (1995) as a trap catch that is more than the additive components of the mixture, and they reported the synergistic ratios (SR). SR is the ratio of the mean trap catch of the mixture (minus the control) to the total mean trap catches of each component in isolation (minus the control). We have calculated these values as well.

Results

Insect Composition and Abundance The primary pollinator trapped was *P. pruinosa* (75% of all bees). *Apis mellifera* (3.3%), *Bombus* spp. (1.7%), *M. bimaculata* (6.6%), *Halictus rubicundus* Christ (Halictidae, 1.7%), *Halictus ligatus* Say (Halictidae, 5%), *Augochlorella striata* Provancher (1.7%), and *Lasioglossum* spp. (5%) were also collected from the traps. *Acalymma vittatum* (62.7% of all beetles) was the primary beetle trapped, followed by *Cyclocephala borealis* (northern masked chafer, Scarabaeidae, 34%) and click beetles (Elateridae, 3.3%).

Beetle Attractants Acalymma vittatum was significantly attracted to T and I (Table 1). There were 2.7 times as many *A. vittatum* caught in traps baited with T compared to traps without T, and traps baited with I were 3.3 times more attractive than those without (Fig. 1a). C was not attractive to *A. vittatum*. The effect of the volatiles on *A. vittatum* attraction was additive; there were no significant interactions. However, the combination of TIC trapped twice as many beetles as T + I + C (SR ratio 2.0; Fig. 2a). Other combinations did not appear to be more attractive than the summation of single components (TI vs. T + I: SR ratio 1.2; TC vs. T + C: SR ratio 0.4; IC vs. I + C: SR ratio 0.1; see Fig. 2a for visual comparisons).

Pollinator Attractants Peponapis pruinosa was significantly attracted to T and C, but not to I (Table 2, Fig. 1b). Traps baited with T attracted 2.7 times more *P. pruinosa* than those without T, and traps baited with C were 3.8 times more attractive than those without. There were no interactive effects of any volatiles on pollinator attraction, and the SR ratio was between 0.4 and 1.1 for all volatile combinations (Fig. 2b).

Table 1 Effects of floral volatiles on the number of *A. vittatum* collected in traps

Source	df	SS	*F*	*P*[a]
T	1	6.53	15.05	**<0.001**
I	1	8.79	20.27	**<0.001**
C	1	0.01	0.02	0.888
T × I	1	1.11	2.56	0.117
T × C	1	0.09	0.21	0.649
I × C	1	0.34	0.78	0.382
T × I × C	1	0.44	1.02	0.319
Block	2	1.32	1.52	0.230
Date	1	10.89	25.12	**<0.001**
Error	43	18.65	–	–

[a] Significant *P* values are shown in bold.

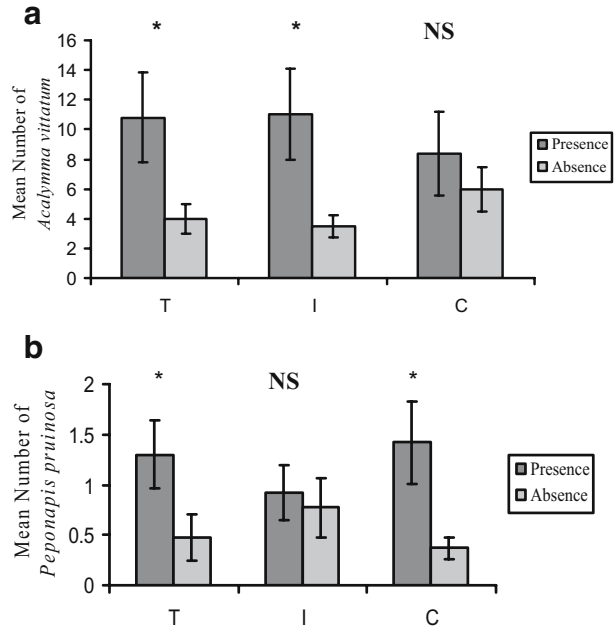


Fig. 1 Mean number of *A. vittatum* (**a**) and *P. pruinosa* (**b**) collected per trap with each volatile treatment. Significant data at $P<0.05$ are indicated by *asterisks*; non-significant data are indicated by *NS*. *Error bars* represent standard error

Discussion

We found that each of the three volatiles tested had different effects on the attraction of pollinators and herbivores. (*E*)-cinnamaldehyde was attractive to the pollinator *P. pruinosa* but not the herbivore *A. vittatum*, indole was attractive to *A. vittatum* but not *P. pruinosa*, and 1, 2, 4-trimethoxybenzene was attractive to both (Fig. 1). Because *Cucurbita* rely on pollinators for reproduction (McGregor 1976), the attraction of pollinators has fitness consequences. Furthermore, *A. vittatum* and related diabroticite beetles can have

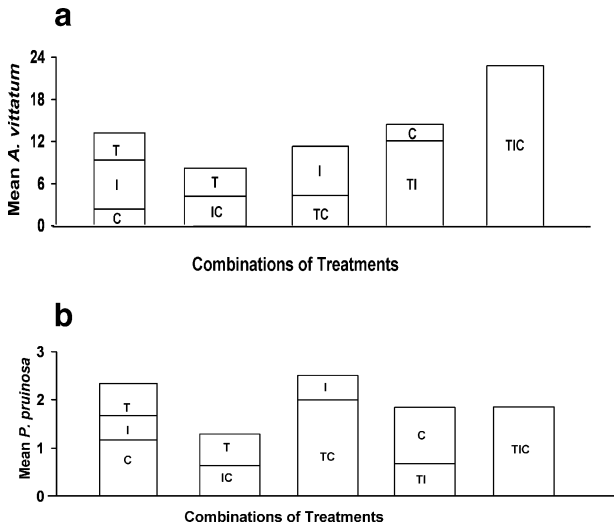


Fig. 2 Number of *A. vittatum* (**a**) and *P. pruinosa* (**b**) caught in traps baited with single volatiles (T, I, or C) as compared to combinations (T + IC, C + TI, I + TC, or TIC together) to illustrate potential synergistic effects

Table 2 Effects of floral volatiles on the number of *P. pruinosa* collected in traps

Source	df	SS	*F*	*P*[a]
T	1	6.87	4.64	**0.037**
I	1	0.003	0.00	0.960
C	1	12.36	8.35	**0.006**
T × I	1	0.9	0.61	0.440
T × C	1	0.6	0.41	0.527
I × C	1	0.36	0.24	0.626
T × I × C	1	0.23	0.16	0.696
Block	2	2.94	0.99	0.379
Date	1	17.46	11.79	**0.001**
Mean position	1	12.3	8.31	**0.006**
Error	42	16.18	–	–

[a] Significant *P* values are shown in bold.

devastating effects on cucurbit growth, reproduction, and survival (Metcalf and Metcalf 1992; Hoffmann et al. 1996). Thus, the attraction of both pollinators and cucumber beetles is likely to have fitness consequences for native cucurbits and implications for crop yield in managed cucurbit systems.

While a growing number of studies have demonstrated that floral display or reward can be costly due to the attraction of antagonists (Brody and Mitchell 1997; Adler and Bronstein 2004; Ashman et al. 2004b; Strauss and Irwin 2004), the current study is one of few to address the role of scent in attracting pollinators compared to herbivores. Similar to our study, specific floral volatiles present in *C. arvense* scent attracted just pollinators, just herbivores, or both pollinators and herbivores (Theis 2006). In addition to having two different corolla shapes, *P. viscosum* has two distinct scent morphs that attract different types of pollinators depending on the presence of floral antagonists (Galen et al. 1987; Galen and Cuba 2001). In areas with low populations of nectar-robbing ants, *P. viscosum* flowers have a wide corolla and a sweet scent that attracts bumblebee pollinators. In areas with high ant populations, flowers have a narrow corolla that deters ants, at the cost of attracting bumblebee pollinators. These flowers emit a skunky scent that attracts fly pollinators that can fit in the narrow corolla.

We found that *A. vittatum* was attracted to 1,2,4-trimethoxybenzene and indole, but not (*E*)-cinnamaldehyde, and that there were no interactive effects of volatiles on attraction. This is contrary to what was observed by Lewis et al. (1990), who found in a field study in Illinois that *A. vittatum* was attracted to indole and (*E*)-cinnamaldehyde, but not 1,2,4-trimethoxybenzene. Similar to their result, we found the combination of TIC to be more attractive than the sum of the parts, with an SR ratio of 2.0. However, our statistical analysis (factorial rather than one-way ANOVA) suggests that this difference is not significant. There are a number of potential explanations for the disparity between our results and those of Lewis et al. (1990). For example, there are many differences in habitat, including microclimate, soil type, and vegetation, between Massachusetts and Illinois. Experimental outcomes can vary over space and time (e.g., Thompson and Cunningham 2002), and the differences between our results and those of Lewis et al. (1990) suggests that this may be the case for the response of *A. vittatum* to *Cucurbita* floral volatiles.

While this research was conducted in an agricultural setting, our results could be relevant to wild cucurbit systems. Native *Cucurbita* species, such as *C. pepo* subsp. *texana* in the southwestern USA, are pollinated by squash and gourd bees of the genus *Peponapis* (Hurd et al. 1971) and are fed upon by *A. vittatum* and other species of diabroticite beetles (Quesada et al. 1995). Pollinators and herbivores could exert different selective pressures on

floral scent, which may influence the evolution of scent composition. Further research could involve manipulative field experiments with *C. pepo* subsp. *texana* in its native habitat to determine if changes in scent volatiles cause appreciable differences in herbivory, pollination, and plant reproduction. Such research should also address the role of scent concentration, which was relatively high in our study, to determine if lower levels that reflect natural blossom emission have similar effects on attraction of both pollinators and herbivores.

Our findings could have important implications for the cucurbit industry. *Cucurbita* fragrance components were not attractive to all visiting specialist insects. For example, we found that only pollinators are attracted to (*E*)-cinnamaldehyde and only herbivores were attracted to indole. The next step would be to determine whether incremental changes in these volatiles lead to differential attraction of pollinators or herbivores. However, there could be unanticipated consequences of a manipulation in volatile levels if these compounds have important functions in other plant parts (e.g., De Moraes et al. 1998). On the other hand, if variation in scent led to increases in pollination or decreases in damage, plant breeders might benefit from selecting plants that produce more (*E*)-cinnamaldehyde or less indole. Decreasing herbivory without the use of pesticides might have the added benefit of limiting damage to local bee populations (Allen-Wardell et al. 1998).

Acknowledgements We thank T'ai Roulston of the Department of Environmental Sciences at the University of Virginia for identifying pollinators, Araujo Farms in Dighton, MA, for the use of their butternut squash field, and S. Halpern, T. Roulston, and two anonymous reviewers for comments on the manuscript. This research was supported by a University of Massachusetts Amherst senior honors research grant awarded by the Commonwealth College to ESA, the National Science Foundation DEB 0542819 to NT, and a Northeastern Integrated Pest Management USDA grant to LSA.

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Introduction

The glassy-winged sharpshooter (GWSS) *Homalodisca vitripennis* (Germar), formerly known as *H. coagulata* (Say), is an important insect pest in California. This pest is native to the southeastern USA and northeastern Mexico (Turner and Pollard 1959) and was first introduced to California in the late 1980s (Sorensen and Gill 1996). Since then, GWSS has established throughout much of California including the San Joaquin, Temecula, and Coachella valleys (Purcell and Saunders 1999; CDFA 2005). GWSS is an important vector of *Xylella fastidiosa* (Wells et al. 1987), a xylem-limited plant pathogenic bacterium that causes Pierce's disease (PD) in grapes, leaf scorches in oleanders, almonds, and plums and many other plant diseases (Hopkins 1989; Blua et al. 1999; Purcell et al. 1999; Hopkins and Purcell 2002). GWSS and the pathogens it carries pose a serious threat to the California viticulture industry, as well as to other horticultural crops throughout the state. For example, in the Temecula valley alone, wineries lost 20 to 30% of their vines to PD between 1997 and 2000 (Hix 2001). Currently, there are no effective cures for *X. fastidiosa*-infected plants. Vector control remains the most important strategy for managing PD and other diseases caused by *X. fastidiosa*.

GWSS feeds on xylem fluid of over 100 plant species in at least 37 families (Turner and Pollard 1959; Adlerz 1980; Hoddle et al. 2003; Redak et al. 2004). One of its major crop hosts in California is citrus. High populations of GWSS were shown to cause stress in orange trees, resulting in reduced yield, fruit size, and quality (Hix et al. 2004). In addition, citrus growers have been hampered by state quarantine regulations that restrict movement of bulk citrus produce and nursery stock infested with GWSS into uninfested areas. Furthermore, GWSS may transmit the PD strain of *X. fastidiosa* into citrus plants that may then serve as reservoirs of the bacterium for further acquisition and transmission to grapevines (Bi et al. 2007). It is known that xylem fluid of citrus plants supports growth and development of *X. fastidiosa* when added to culture media (Bi et al. 2007). Improved understanding of the nutritional ecology of GWSS on citrus may help to develop better control strategies.

Numerous studies on nutritional ecology have demonstrated the importance of nitrogen nutrition on survival, growth, and reproduction of phytophagous insects (e.g., Mattson 1980; White 1984; Bi and Felton 1995; Bi et al. 1994, 1997, 2001, 2003, 2005a; Simpson et al. 1995; Joern and Behmer 1997; Blackmer and Byrne 1999). The primary nitrogen nutrients in xylem fluid of GWSS host plants are amino acids and soluble proteins (Andersen et al. 1989, 1992; Bi et al. 2005b). However, xylem fluid is the most dilute food source known for herbivores and requires that strict xylophages such as GWSS engage in exceptionally high feeding rates to acquire sufficient nutrition (Strong et al. 1984; Andersen et al. 1989, 1992). In addition, specialized endosymbiotic bacteria also contribute essential amino acids to their xylem-feeding insect hosts (Wu et al. 2006). In the case of GWSS, two different species of endosymbionts are found compared to one species in phloem feeding insects such as aphids and whiteflies, suggesting a greater requirement for the xylophage to augment incomplete dietary sources through metabolically derived amino acids provided by endosymbionts (Wu et al. 2006). Regardless of the supplemental amino acids provided by endosymbionts, however, complete nutrition for GWSS requires that dietary intake be sufficient to meet demands of growth and reproduction while maintaining energy at adequate levels for host seeking and finding.

As a year-round host in southern California, citrus plays a critical role in the growth and reproduction of GWSS. We previously studied the influence of seasonal nitrogen nutrition fluctuations on the population dynamics of GWSS in citrus (Bi et al. 2005b). In particular,

shifting densities of GWSS adults between lemon and orange trees were correlated to changes in concentrations of certain xylem fluid amino acids on three different occasions during the year-long study. In each case, significant increases in density were associated with positive changes in dietary nitrogen as represented by the free amino acids, not soluble proteins (Bi et al. 2005b). The large fluctuations in amino acid concentrations throughout the annual cycle in both lemon and orange were revealing with respect to how host plants vary temporally in nutritive quality. Coupled with data on shifting densities of GWSS between lemon and orange, the seasonal variability in amino acid concentrations helped to provide greater insight into GWSS movement and host shifts. Previous studies that demonstrated the essential role played by particular amino acids in GWSS ecology (Brodbeck et al. 1990, 1999, 2004; Andersen et al. 1992, 2003, 2005) add support to the conclusion that the observed redistributions between lemon and orange were triggered by changing amino acid concentrations (Bi et al. 2005b).

In addition to occasional shifts among host plants that occur in GWSS feeding ecology, other patterns of GWSS host utilization have been observed but not documented. One of the most prominent of these patterns is the propensity of GWSS adults to feed in higher densities on younger citrus trees relative to older ones. What has not been clear is whether the pattern of higher densities on younger trees has been because of species or varietal differences between the young and old trees rather than to age of the tree itself. We decided to investigate this phenomenon more closely by comparing GWSS densities on a stand of young citrus trees growing adjacent to older trees of the same species and variety. In addition to measuring potential differences in GWSS densities on young vs. old trees, we also were interested in looking for a nutritional basis for such differences. The present study was initiated to determine the influence of seasonal amino acid fluctuations in xylem fluid on GWSS population densities on young and old orange trees.

Methods and Materials

Experimental Plots An orange grove at Newhall Ranch, Piru, Ventura county of California was the site for experiment. Commercially grown young and old orange trees (*Citrus sinensis* var. Valencia grafted on Carrizo) in adjacent blocks were used, separated only by a 3-m access road. The young trees were 6 years old, whereas the old trees were 30 years old. There were more vigorously growing shoots in the young than in the old trees. Twenty trees were randomly selected from each block to monitor GWSS population densities, and 15 of them were used to extract xylem fluid.

Sampling of GWSS A bucket-sampling device (5-gal plastic bucket, with a funneling collecting jar at the bottom, attached to an extendable pole) as described by Castle et al. (2005a) was used to sample both immature and adult GWSS in young and old orange trees. Seven beats were processed with the bucket in each of five directions (360°/5) around the trees. Adults and nymphs in the collecting jar from each tree were transferred to ziplock plastic bags and returned to the laboratory for counting. Population densities of both adult and immature GWSS were monitored on a biweekly basis.

Collection of Xylem Fluid Xylem fluid was used for determination of nutritional quality because GWSS exclusively feeds on xylem fluid (Andersen et al. 1989). Samples of fluid were collected between 6:00 a.m. and 10:00 a.m. on biweekly sampling dates by using a pressure chamber apparatus (Andersen et al. 1989, 1992). One to three terminal shoots from

each tree were used for xylem fluid extraction because these shoots are the preferred feeding sites for GWSS on citrus plants. Upon collection, the xylem fluid was immediately placed on dry ice before final storage in a −80°C freezer. The samples were used for chemical analyses of free amino acids. Xylem fluid sampling was initiated on 3 September 2002 and terminated on 10 September 2003.

Determination of Free Amino Acids Free amino acids were quantified with a Perkin Elmer Applied Biosystem Model 420A PTC derivatizer with an on-line Perkin Elmer Applied Biosystem Model 130A PTC Amino Acid Analyzer. After filtration with a 0.45-μm syringe filter, 20 μl of xylem fluid were reacted with phenylisothiocynate to produce phenylthiocarbamyl (PTC) amino acid derivatives. After derivatization, a methanol solution containing the PTC-amino acids was transferred to a high-performance liquid chromatography system for separation. The PTC-amino acids were separated on a Spheri-5 PTC column (220×2.1 mm), and the PTC chromophore was detected at 263 nm. The buffer system used for separation was 50 mM sodium acetate pH 5.45 as buffer A and 70% acetonitrile/32 mM sodium phosphate pH 6.1 as buffer B. The program was run by using a gradient of buffer A and buffer B with an initial 7% buffer B concentration and ending with a 60% buffer B concentration at the end of the gradient. A standard mixture of known quantities of individual amino acids (Sigma Chemical Co.) was derivatized as above and used for quantification and identification of individual amino acids.

Statistical Analyses A repeated measures analysis of variance (ANOVA; SAS Institute Inc 1989) was used to analyze densities of GWSS during the period when they were present to compare densities on young vs. old trees. Numbers of GWSS adults from bucket-collected samples were $\log(y+1)$ transformed before ANOVA to normalize the data (Little and Hill 1978). Concentrations of individual amino acids in young and old trees during the period when GWSS present or absent were also analyzed by repeated measures ANOVA (SAS Institute Inc 1989). A separate repeated measures ANOVA was conducted on amino acid concentrations during the interval that GWSS was absent. Principal component analysis followed by factor analysis (SAS Institute Inc 1989) was run separately for young and old orange data sets to identify groups of amino acids that exhibited similar dynamical behavior. Outlier analysis by using Mahalanobis distances was applied before factor analysis to eliminate outlying points from the analysis. Individual amino acids with loading values $>|0.7|$ within each factor were grouped, and their annual concentration profiles were presented graphically.

Results

GWSS Densities Differences in GWSS densities relative to amino acid concentrations were evaluated only over the first ten sampling dates when GWSS was present. Beginning with the first sampling on 1 September 2002, higher numbers of adult GWSS occurred on young trees compared to old trees. This difference became increasingly pronounced on each successive sampling date through 22 October 2002 (Fig. 1). After a cold day in early November 2002, GWSS numbers rebounded moderately with consistently higher numbers on young trees compared to old trees through 4 February 2003. After this date, negligible numbers of GWSS (<1 per tree, data not shown) were caught despite conducting the same

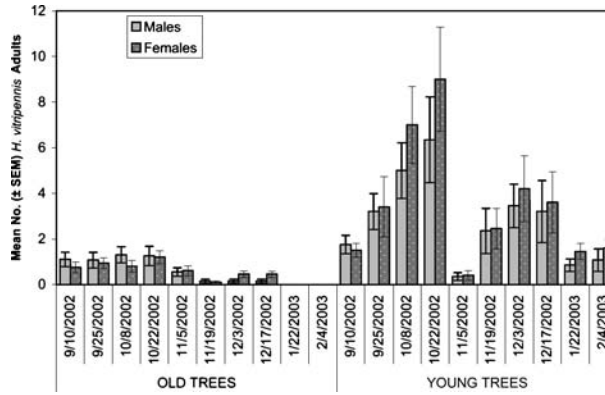


Fig. 1 Mean (±SE) numbers of *H. Vitripennis* adult males and females in young and old orange trees in southern California between September 2002 and February 2003

sampling routine for the remainder of the study. Significantly greater numbers ($F_{(1,28)}=54.45$, $P<0.001$) of adult GWSS occurred on young vs. old orange trees during the September 2002–February 2003 period. Few nymphs were caught during this time (<1 nymphs per tree, data not shown), consistent with previous findings on the phenology of GWSS in citrus (Castle et al. 2005b). There was a slight trend of higher numbers of females on young trees compared to males, with the converse being true for old trees (Fig. 1).

Free Amino Acids Biochemical analyses of xylem fluid sampled from young and old orange trees revealed 19 amino acids present in most samples collected throughout the year. Concentrations of individual amino acids ranged widely relative to one another during either period of the year marked by the presence or absence of GWSS (Tables 1 and 2). Mean concentrations of 12 amino acids including asparagine, serine, glutamine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine were significantly higher in young trees compared to old trees during the time that GWSS was present (Table 1). These amino acids together with glycine, arginine, alanine, proline, and cysteine were significantly higher in the next period after the disappearance of GWSS (Table 2). The same was true for the mean totals of nine essential amino acids for insect growth and development that includes isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, arginine, and histidine (Taylor and Medici 1966; Brodbeck and Strong 1987; Tables 1 and 2). Similar to previous findings (Bi et al. 2005b), concentrations of proline in orange trees far exceeded any other amino acid with individual sampling date means ranging year-long from 154–463 nmol ml^{-1} in young trees and 99–409 nmol ml^{-1} in old trees (Tables 1 and 2). Apart from double digit mean concentrations of asparagine, glutamine, serine, and threonine occurring during one or both periods, all other amino acids had mean concentrations <10 nmol ml^{-1} (Tables 1 and 2).

Factor analysis revealed commonalities among various amino acids that also were apparent in concentration profiles of amino acids collected over the 1-year period. Separate analyses of young and old orange trees produced rotated loading matrices that included three factors (eigenvalues > 1) for each age category (Table 3). Factor 1 for young trees accounted for 59.6% of the variance in the analysis and contained eight amino acids (asparagine, serine, glutamine, histidine, threonine, tyrosine, valine, and lysine) that had loading values > |0.7|. Another 11.8 and 10.2% of the variance was accounted for by factors 2 (contained aspartic acid, glutamic acid, glycine, and arginine) and 3 (contained leucine and phenylalanine), respectively, for a cumulative 81.6% for all three factors that included

Table 1 Comparison of seasonal means of amino acids during the time period *Homalodisca vitripennis* present on experimental trees (from 10 September 2002 to 02 February 2003)

	Young Tree (nmol ml^{-1} xylem fluid)	Old Tree (nmol ml^{-1} xylem fluid)	*F*	*P*
Aspartic Acid	3.25 (0.46–11.90)	3.29 (0.62–12.94)	4.11	0.06
Glutamic Acid	6.99 (1.12–19.57)	8.38 (2.61–27.32)	0.13	0.73
Asparagine	*25.83* (9.44–53.47)	15.76 (7.79–23.07)	12.93	<0.01
Serine	*12.59* (5.04–23.46)	9.21 (3.58–23.58)	10.69	<0.01
Glutamine	*15.41* (4.52–29.26)	7.91 (3.44–12.48)	15.21	<0.01
Glycine	3.33 (1.09–7.37)	3.72 (1.45–5.66)	0.92	0.35
Histidine	*0.55* (0.00–1.54)	0.33 (0.00–0.89)	12.81	<0.01
Arginine	5.62 (0.83–13.88)	4.82 (2.02–8.78)	0.39	0.54
Threonine	*8.99* (3.67–15.94)	3.82 (2.50–6.46)	37.83	<0.01
Alanine	6.87 (3.56–9.99)	6.70 (4.38–9.20)	0.01	0.93
Proline	318.17 (180.0–463.4)	291.08 (230.9–386.5)	1.69	0.21
Tyrosine	*1.97* (1.38–2.95)	1.30 (0.94–1.56)	33.16	<0.01
Valine	*8.13* (4.98–12.30)	4.80 (2.98–6.60)	28.17	<0.01
Methionine	*2.08* (1.06–4.02)	1.03 (0.31–1.93)	54.37	<0.01
Cysteine	4.99 (0.22–13.56)	5.60 (0.13–18.39)	0.44	0.52
Isoleucine	*4.02* (2.51–5.99)	2.30 (1.55–2.99)	38.39	<0.01
Leucine	*4.77* (3.25–9.32)	2.75 (1.78–3.39)	72.29	<0.01
Phenylalanine	*2.61* (1.79–5.23)	1.61 (1.27–1.91)	43.10	<0.01
Lysine	*1.45* (0.43–3.12)	0.69 (0.19–1.38)	27.28	<0.01
Essential AAs	*38.22* (22.10–70.72)	22.13 (18.04–26.48)	28.59	<0.01

Italicized values denote significantly greater quantities of respective amino acids in comparison of young vs. old trees.

14 amino acids with loading values > |0.7|. For old trees, a cumulative 78.9% of the factor analysis solution was explained by the first three factors that contained a total of 17 amino acids with loading values > |0.7|.

Higher concentrations of total essential amino acids were observed in young trees on nearly all of the 24 sampling dates with differences especially pronounced during fall and late spring–early summer (Fig. 2). The steep rise in the essential amino acids in young trees on the first four sampling dates (2 September–22 October 2002) paralleled the steep rise in GWSS numbers on young trees over the same time period. In contrast, levels of the essential amino acids and GWSS numbers remained flat in old trees during this period. The eight amino acids contained in factor 1 for young trees were characterized by concentration profiles similar to the composite profile for essential amino acids, especially with respect to elevated concentrations in young trees during late spring–early summer (Fig. 3). During the first part of the sampling period when GWSS was present, significantly higher concentrations for all eight amino acids occurred in young trees. However, the age of orange trees was not a significant factor for those amino acids contained in factor 2 for young trees: aspartic acid, glutamic acid, arginine, and glycine (Table 3; Fig. 4). The concentrations of aspartic acid and glutamic acid were similar to one another in young and old trees with reduced concentrations observed from November through April and increased concentrations during warmer months. Peak concentrations for arginine and glycine were observed during early fall with elevated levels occurring as late as 22 January 2003 before declining through late winter–mid spring (Fig. 4).

Table 2 Comparison of seasonal means of amino acids during the time period *Homalodisca vitripennis* absent on experimental trees (from 17 February to 02 September 2003)

	Young (nmol ml^{-1} xylem fluid)	Old (nmol ml^{-1} xylem fluid)	*F*	*P*
Aspartic Acid	2.94 (0.00–19.12)	3.34 (0.00–16.41)	4.84	0.04
Glutamic Acid	7.00 (0.00–52.47)	6.79 (0.00–33.08)	0.08	0.78
Asparagine	*65.53* (0.00–379.35)	28.65 (0.00–227.59)	39.08	<0.01
Serine	*14.00* (0.00–83.64)	11.28 (0.00–55.29)	7.04	0.01
Glutamine	*31.61* (0.00–224.80)	14.32 (0.00–122.86)	43.78	<0.01
Glycine	*2.43* (0.00–25.14)	1.74 (0.00–6.13)	17.82	<0.01
Histidine	*1.27* (0.00–8.34)	0.61 (0.00–5.98)	67.74	<0.01
Arginine	*1.92* (0.00–9.70)	1.15 (0.00–6.05)	79.26	<0.01
Threonine	*14.29* (0.00–76.48)	8.24 (0.00–62.60)	37.09	<0.01
Alanine	*6.12* (0.55–13.06)	5.02 (0.69–13.46)	20.15	<0.01
Proline	*284.47* (38.64–492.52)	218.28 (31.95–499.15)	42.18	<0.01
Tyrosine	*2.25* (0.20–10.17)	1.40 (0.20–5.73)	58.33	<0.01
Valine	*8.94* (0.34–37.98)	6.14 (0.00–31.63)	24.75	<0.01
Methionine	*2.45* (0.00–12.38)	1.51 (0.00–7.69)	34.38	<0.01
Cysteine	*5.23* (0.00–22.69)	4.41 (0.00–33.54)	6.93	0.02
Isoleucine	*4.31* (0.00–17.20)	3.11 (0.00–15.49)	19.29	<0.01
Leucine	*3.44* (0.35–11.95)	2.65 (0.28–11.15)	31.66	<0.01
Phenylalanine	*2.00* (0.00–6.94)	1.43 (0.00–6.08)	29.76	<0.01
Lysine	*1.86* (0.00–13.85)	0.81 (0.00–3.54)	171.40	<0.01
Essential Aas	*40.16* (0.77–182.96)	25.31 (1.20–122.96)	22.01	<0.01

Italicized values denote significantly greater quantities of respective amino acids in comparison of young vs. old trees.

Concentration peaks that occurred for some amino acids in young trees were often attenuated or absent in old trees. This was most apparent in leucine and phenylalanine in which mid to late October peaks in young trees were missing in old trees (Fig. 5). Peak concentrations of isoleucine and methionine tended to be more tri-modal in young trees than old trees, occurring in fall, early spring, and early summer (Fig. 5). Similar tri-modal profiles, although with greater variation, were also observed for proline and alanine (Fig. 6). Cysteine concentration in young and old trees was the most erratic of any amino acid, being virtually absent from samples on some dates and in relatively high concentrations on surrounding dates (Fig. 6).

Discussion

Successful growth and maintenance of GWSS populations often requires host switching among the many plant species upon which GWSS feeds (Mizell and French 1987; Brodbeck et al. 1999; Redak et al. 2004). At the beginning of this field study in September 2002, GWSS appeared to be relatively abundant in the experimental orchard and surrounding area. In addition, conspicuous numbers were spotted on vegetation in a riparian zone that traversed through the ranch property. Increasing densities of GWSS adults on the young orange trees through the end of October may have reflected host shifting from vegetation outside the orchard. During this same period, however, very little

Table 3 Factor loading values for amino acids in xylem fluid from young and old orange trees collected over a 1-year period

	Factor					
	Young Trees			Old Trees		
Amino acids	1	2	3	1	2	3
Aspartic Acid	0.385	*0.841*	−0.181	−0.011	0.498	*0.775*
Glutamic Acid	0.321	*0.870*	−0.162	−0.059	0.575	*0.713*
Asparagine	*0.927*	0.189	0.024	*0.773*	0.010	0.514
Serine	*0.717*	0.603	0.179	0.376	0.297	*0.795*
Glutamine	*0.929*	0.165	0.133	*0.820*	0.021	0.430
Glycine	0.144	*0.767*	0.425	0.076	*0.861*	0.087
Histidine	*0.849*	0.227	0.267	*0.814*	0.076	0.214
Arginine	−0.036	*0.739*	0.356	0.010	*0.794*	0.099
Threonine	*0.905*	0.282	0.224	*0.782*	0.030	0.569
Alanine	0.325	0.576	0.479	0.347	*0.771*	0.267
Proline	0.371	0.484	0.562	0.506	*0.709*	0.091
Tyrosine	*0.724*	0.335	0.471	*0.797*	0.253	0.351
Valine	*0.753*	0.189	0.586	*0.942*	0.172	−0.025
Methionine	0.610	−0.073	0.609	*0.855*	0.092	−0.153
Cysteine	0.005	−0.102	0.596	0.174	0.282	−0.483
Isoleucine	0.644	0.142	0.662	*0.901*	0.129	−0.151
Leucine	0.256	0.391	*0.809*	0.757	0.416	−0.228
Phenylalanine	0.389	0.446	*0.739*	0.789	0.473	0.031
Lysine	*0.700*	0.557	0.225	0.375	0.530	0.601
Eigenvalue	11.330	2.240	1.939	9.589	3.354	2.053
Percent	59.6	11.8	10.2	50.5	17.7	10.8
Cumulative %	59.6	71.4	81.6	50.5	68.1	78.9

Loading values > | 0.7 | are italicized

change in GWSS densities was observed on the old orange trees in spite of their proximity to the young trees. Thus, GWSS adults immigration into the experimental orchard were responding positively to the young trees while showing no net change in densities on the old trees.

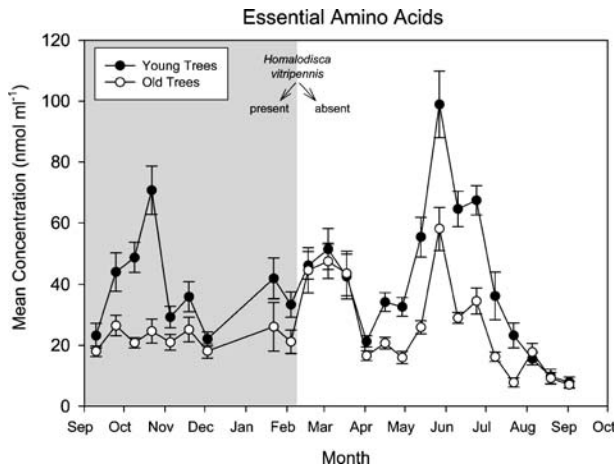


Fig. 2 Mean (±SE) concentrations of the total essential amino acids from September 2002 through September 2003 in young and old orange trees. *Shaded area* represents the period when GWSS was present

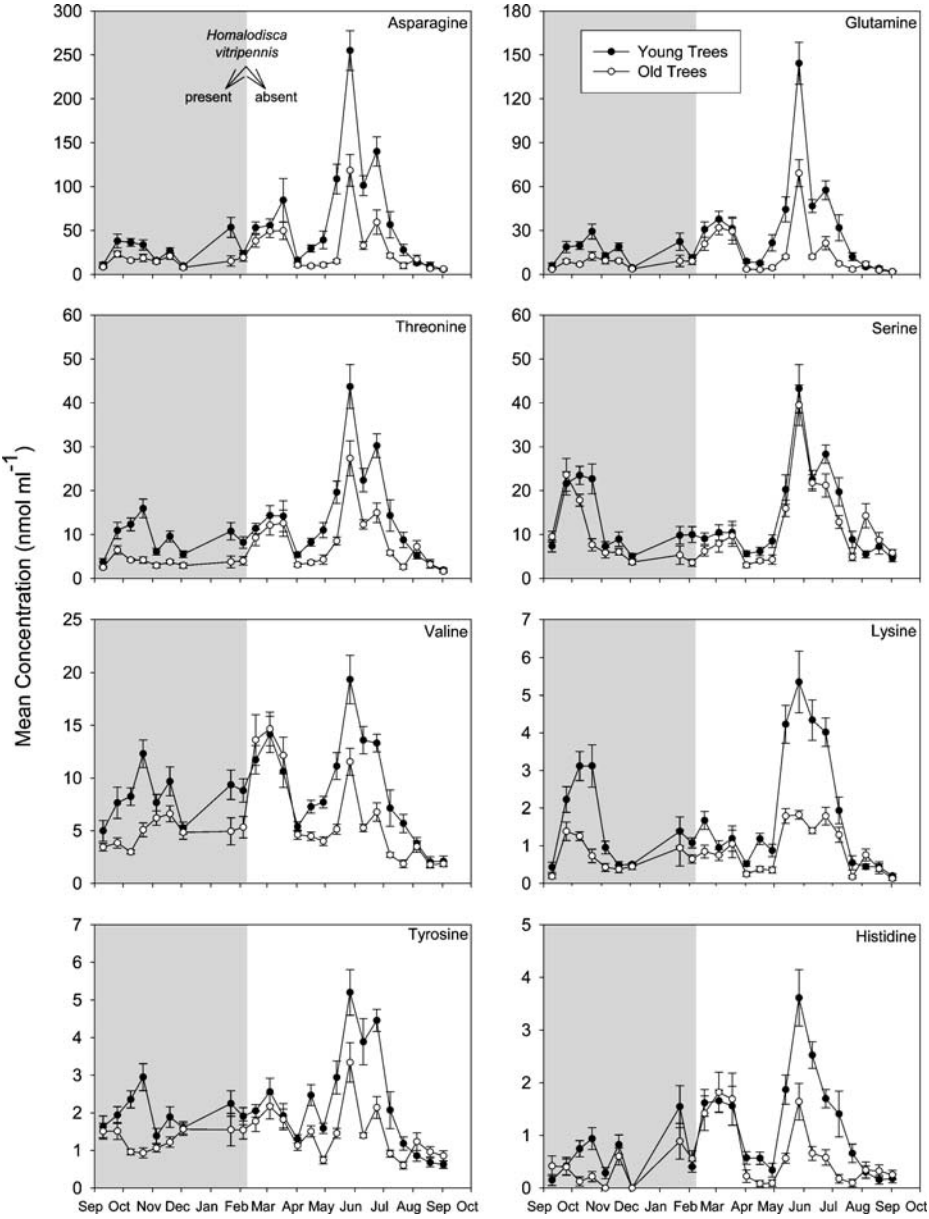


Fig. 3 Annual concentration profiles of eight amino acids from xylem fluid of young or old trees that loaded onto factor 1 in the young tree analysis

At the same time that GWSS adult densities were increasing on young orange trees, concentrations of most amino acids including all nine essential amino acids also were increasing. Importantly, elevated amino acid concentrations observed during the September–October 2002 interval occurred principally in young trees, whereas remaining static or inferior in old trees. Higher levels of asparagine, arginine, proline, glutamine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, and serine

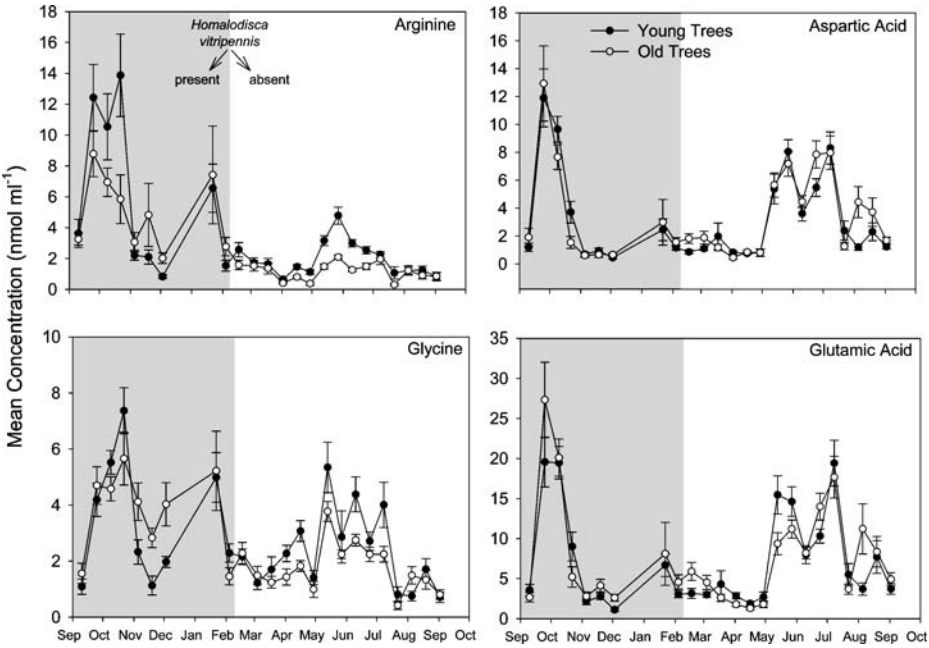


Fig. 4 Annual concentration profiles of four amino acids from xylem fluid of young or old trees that loaded onto factor 2 of the young tree analysis

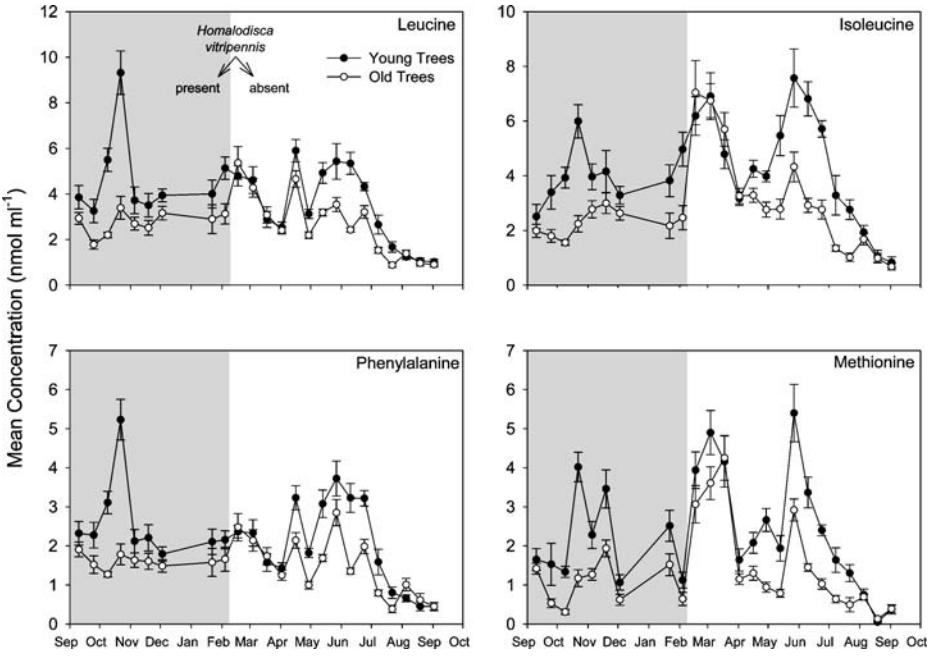


Fig. 5 Annual concentration profiles of four of the ten amino acids from xylem fluid of young or old trees that loaded onto factor 1 of the old tree analysis

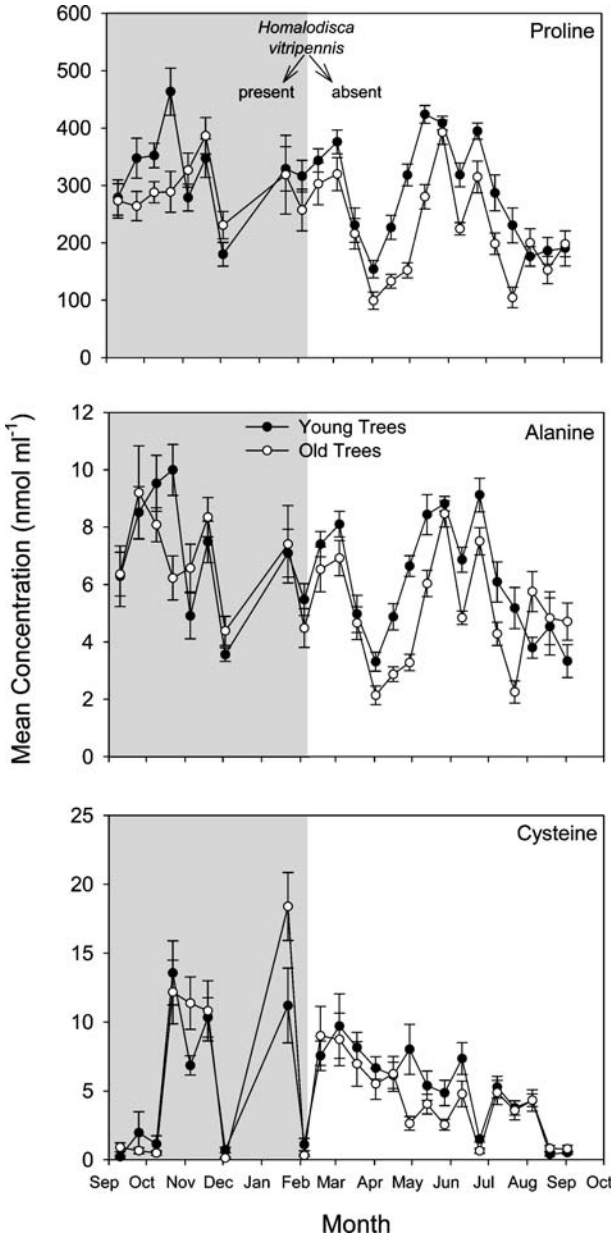


Fig. 6 Annual concentration profiles of proline and alanine from xylem fluid of young or old trees that loaded onto factor 2 of the old tree analysis and of cysteine that did not load into any factor of the young or old tree analyses

were determined in the xylem fluid of young orange trees. Additionally, concentration peaks of glycine, alanine, and cysteine varied between young and old trees, but all 14 remaining amino acids attained significantly higher concentrations in young trees than old trees during the period of GWSS adult recruitment to young trees in fall 2002. Peak densities of GWSS adults were observed on 22 October 2002, after which cooler temperatures and attrition resulted in fewer GWSS adults overall, but with consistently higher numbers remaining in young trees through 04 February 2003. After this date, virtual disappearance of the GWSS

population precluded comparison of GWSS densities on old vs. young trees. However, a similar pattern of significantly higher concentrations of amino acids in young trees compared to old trees continued through the spring and summer seasons before terminating xylem fluid sampling in September 2003.

The propensity of GWSS to feed upon young trees preferentially to old trees observed in this study coincide with general observations of higher GWSS densities found on younger citrus trees rather than older trees. Researchers in California have learned to seek out younger citrus trees for collecting GWSS specimens, but often it is unclear whether species or varietal differences play a role or whether agronomic factors such as fertilization or irrigation regimes potentially affect distribution of GWSS populations. The experimental site in the present study was carefully chosen so as to eliminate most variables other than tree age in terms of GWSS host selection. Both young and old experimental trees were the same variety (Valencia) and under the same irrigation schedule. Their respective positions in adjacent blocks ensured exposure to the same microenvironmental climate. The most significant difference between the young and old trees was the nutritional quality of their xylem fluid as determined by the measurement of 19 amino acids over a 1-year period. The consistently higher concentration of essential amino acids and most of the remaining amino acids in young trees in conjunction with much higher GWSS densities feeding on the young trees strongly support the nutritional superiority of young citrus trees over old trees. The consistently higher densities on young trees also point to the capability of GWSS to locate nutritionally superior trees and exploit them for as long as the nutritional advantage is sustained.

Host switching enables the highly mobile GWSS adults to locate and feed upon nutritionally superior plants. Carbon nutrition may be abundant for GWSS in xylem fluid of host plants such as grapes and citrus because the abundance of GWSS on these plants is not consistently correlated to levels of sugars in the xylem fluid (Andersen et al. 2005; Bi and Toscano, unpublished data), whereas nitrogen nutrition plays a more important role in GWSS host selection (Andersen et al. 2005; Bi et al. 2005b). The primary nitrogen nutrients in xylem fluid of GWSS host plants are amino acids and soluble proteins (Andersen et al. 1989, 1992; Bi et al. 2005b). Soluble protein levels in oranges and lemons did not correspond to shifting GWSS numbers as did amino acids (Bi et al. 2005b). GWSS host selection among *L. indica*, *B. halimifolia*, peach (*Prunus persica*), and plum (*P. salinicia*) was positively correlated with levels of asparagine and glutamine in xylem fluid (Brodbeck et al. 1990). GWSS abundance on grape (*Vitis*) genotypes was correlated to the levels of glutamine, methionine, and phenylalanine (Andersen et al. 2005). We previously reported that GWSS host switching from lemon to orange was closely associated to increase in 12 amino acids in orange including asparagine, glutamine, histidine, threonine, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, and glycine (Bi et al. 2005b). Glutamine and asparagine may be ideal host selection cues for generalist feeder GWSS (Brodbeck et al. 1990). These amides are also considered phagostimulants for GWSS (Andersen et al. 1992). Tyrosine and phenylalanine are essential for insect cuticle formation (Bernays and Woodhead 1984). Results in this study provide further evidence that GWSS host selection is dependent upon levels of amino acids in xylem fluid (Andersen et al. 2005).

The drastic decline in GWSS numbers on 5 November 2002 occurred in conjunction with a severe temperature drop. The minimum temperature on that sampling date was below 10°C (data not shown). We noticed that many GWSS had dropped to the ground especially from the young trees, resulting in the lower mean numbers for this sample date. The larger canopies of older trees may provide better protection from cold and other

environmental extremes. Johnson et al. (2006) reported the low temperature threshold for GWSS feeding was 10 and 9.6°C lasting for 12.2 days caused 50% mortality of GWSS. The duration of low temperature in this experiment was relatively short (<12 h). A small portion of the GWSS may be killed directly by the cold and/or the cold resulting starvation may make them more vulnerable to be killed by natural enemies.

The modest decrease in GWSS densities observed from November through December probably represented the decline often observed in insect populations in general during conditions of shorter daylength and cooler temperatures. The sudden disappearance of GWSS after February 4, 2003, however, was unexpected based on the pattern established over the previous 5 months in the experimental orchard or relative to GWSS populations observed in other areas of southern California (Castle et al. 2005b). Area-wide management programs implemented against GWSS in various citrus-intensive regions of southern California have proven effective. The area-wide GWSS management program was initiated in Ventura county in 2003 to protect the shipping nurseries. This program involves treating nurseries and the surrounding citrus groves with soil-formulated imidacloprid (Admire, Bayer CropScience). Imidacloprid is persistent in citrus plants and keeps GWSS in control for 4–5 months (Castle et al. 2005a). There were nurseries with surrounding citrus groves, close to our experimental orchard, treated in early 2003 with imidacloprid. GWSS is extremely mobile with the capacity to move over 1 km in a relatively short time (Blua et al. 1999; Blackmer et al. 2004; Park et al. 2006). It has been noticed that in other areas of California, e.g., in the San Joaquin valley, GWSS numbers on surrounding crops were greatly depressed, although the chemical control program targeting GWSS was applied only to citrus (Park et al. 2006). The disappearance of GWSS after March 2003 in this study (Fig. 1) may be caused by the area-wide GWSS management program.

Acknowledgements We thank Dr. Tim Kingan and Mr. Gerald Porter for their technical support and G. R. Ballmer for his assistance in sample collection. This research was funded in part by the University of California Pierce's Disease Control Program.

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(Fabaceae) that are pollinated by passerine birds appear to have higher amino acid concentrations than hummingbird-pollinated *Erythrina* species (Baker and Baker 1982b). According to Cruden and Toledo (1977), this may explain why passerine birds defend territories on flowering *Erythrina* trees. In contrast, Gottsberger et al. (1990, 1984) cautioned against attributing too much significance to measured amino acid concentrations because of the likelihood of their leaching from dislodged pollen grains.

Some older publications concerning nectar amino acids were based on semiquantitative techniques, such as ninhydrin staining and comparison with a histidine scale for total amino acid concentrations or thin-layer chromatography for identifying the individual amino acids (Baker and Baker 1973, 1977). With the use of high-performance liquid chromatography (HPLC), more data have become available for amino acids in nectar, including two large surveys of insect-pollinated flowers; 30 species growing in Britain (Gardener and Gillman 2001a) and 73 species from a single plant community in Greece (Petanidou et al. 2006). Information on the nectar amino acids of bird-pollinated flowers is lacking, however, and the study by Baker and Baker (1982b) on *Erythrina* is a good reason to revisit this genus. *Aloe* is another important genus for avian nectarivores (Oatley and Skead 1972). In addition to surveying *Aloe* and *Erythrina* species, I examined other bird-pollinated genera and looked at two potential sources of variation in nectar amino acids: pollen contamination and the variation within and among individual plants.

Methods and Materials

Nectar collection Nectar was collected from plants growing in the field and in cultivation in various locations in and around Pretoria. These included parks, street plantings, private gardens, school grounds, botanic gardens in Pretoria and Johannesburg, and the Suikerbosrand Nature Reserve (SW of Johannesburg). Many species were sampled in the Pretoria National Botanic Garden and the University of Pretoria's Botanic Garden. Where necessary, identifications were confirmed with the curators of herbaria and botanic gardens. Four South American species of *Erythrina* were also included in the survey. A list of collection locations is on file with the author. Only single analyses are presented for many of the species because of cost considerations and, in some cases, availability of plant material.

Nectar samples of approximately 100 μl were collected with care to avoid contamination with pollen or damage to floral tissue and frozen before analysis. Because bird-pollinated flowers produce large volumes of nectar, it was usually possible to collect from single flowers, but sometimes I pooled nectar from different flowers on an inflorescence or raceme. For all nectar samples, sugar concentrations (% *w*/*w*) were measured at the time of collection with a pocket refractometer (Bellingham & Stanley, Tunbridge Wells, UK).

Amino acid analysis The Pico Tag method was used to measure the concentrations of free amino acids with HPLC, by using precolumn derivatization with phenylisothiocyanate (PITC) (Bidlingmeyer et al. 1984). A reversed-phase column (30 cm Pico Tag, Waters) was used for the separation with solvent A being 0.07 M sodium acetate (pH 6.45) with 2.5% acetonitrile and solvent B consisting of 45% acetonitrile, 15% methanol, and 40% water. The gradient used was: 0% B for 14.5 min; direct change to 3% B; 6% B over 9.5 min with a concave gradient; 9% B over 6 min with a convex gradient; 35% B over 20 min linear; kept at 35% B for 10 min; 100% B linear in 30 sec; 100% B for 5 min. Standards (37 amino acids) were included after every six samples. Amino acids were detected at 254 nm with a Waters model 440 UV absorbance detector, and Millenium32 Chromatography Manager

from Waters was used to calculate peak areas. All samples were run in duplicate. Serine and asparagine could not be reliably resolved and have been combined. Large proline peaks on the chromatograms did not show clear separation from 1-methylhistidine, anserine or 3-methylhistidine. Only data for the common protein amino acids are presented: other amino compounds, such as the three just mentioned or hydroxyproline, carnosine, and ornithine, were present only in trace amounts. Note that the total amino acid concentrations will be slightly underestimated because of unresolved peaks.

Effect of pollen on nectar amino acids Three racemes of *Aloe marlothii* were collected from different plants in the same population at Suikerbosrand Nature Reserve. Nectar from each raceme was divided into two 1 ml samples in 1.5 ml Eppendorf© tubes. One served as a control and 5 mg pollen, brushed from freshly dehisced anthers on the same raceme, was added to the other. The pollen–nectar mixture was immediately vortexed and left to stand for 5 min, then filtered through a 0.2 μm filter before the filtrate was frozen for amino acid analysis. Each nectar sample was analyzed twice to estimate the reliability of the data and allow for an error term to analyze the variance in amino acid composition between the pollen and control treatments.

Variability in nectar amino acid composition The deciduous trees of *Erythrina lysistemon* bear scarlet flowers in early spring before the leaves appear. Nectar was collected from four inflorescences on each of six trees in August–September 2005. This is the dry season in Pretoria, and none of the trees was watered. The amino acid composition of each nectar sample was quantified twice to estimate the reliability of the data and allow for an error term to analyze the variance in amino acid composition among the inflorescences.

Statistics Data are expressed as the means±SE. The effect of added pollen on nectar amino acid composition in *A. marlothii* was analyzed using a repeated measures ANOVA with duplicate measures nested within the two treatments (control and added pollen). The relative partitioning of variance within and between *E. lysistemon* trees was analyzed by ANOVA and by calculating repeatability. To quantify the different hierarchical levels of variation in nectar amino acid composition in *E. lysistemon*, separate nested ANOVA were performed for each amino acid using the PROC NESTED procedure of the SAS V 9.1 statistics package (SAS Institute, Cary, N.C., USA). To examine differences in composition independently of the total concentration, the data were adjusted to a total amino acid concentration of 97.5 mM (the mean value for the 24 inflorescences used to examine variability), and reanalyzed. Each analysis contained three sources of variation, nested as follows: *Duplicate* measures (N=2) nested within *Inflorescence* (N=4), which was nested within *Tree* (N=6). The critical level for statistical analyses was set at $\alpha<0.05$ and the significance of the F values was subjected to a sequential Bonferroni correction (Rice 1989). All F values indicating significance are at Bonferroni probabilities. Repeatability±SE was calculated following Becker (1984).

Results

Survey of nectar amino acids in bird-pollinated flowers Among *Aloe* species, high amino acid concentrations were measured in the nectar of *A. marlothii*, but most other species had relatively low concentrations (Table 1). Undetectable amino acids are shown by blank

Table 1 Amino acids in the nectar of 16 *Aloe* species

Species	*aculeata*	*arborescens*	*barberae*	*castanea*	*ciliaris*	*greatheadii* var *davyana*	*excelsa*	*ferox*		*marlothii*	*pretoriensis*
						(*N*=4)		(1)	(2)		(*N*=4)
ARG	0.29					1.10±0.03				0.11	0.84
HIS											
ILE	0.26		0.2	0.21		0.10	0.97	0.51	0.93	1.68±0.11	
LEU	0.1		0.65	0.14			0.93	0.35	0.85	1.86±0.13	
LYS	0.02		0.83					0.39	0.01	0.30±0.13	
MET	0.1	0.1	0.08	0.41	0.1	0.15	0.39	0.24	0.2	0.59±0.13	0.08
PHE	2.29		0.52	0.66			1.2	0.44	1.35	1.91±0.35	
THR	0.22					0.15	0.88	0.49	0.72	1.39±0.33	
TRP	0.21		0.15	0.11			0.2	0.19	0.18	0.24±0.03	
VAL	0.56	0.01	0.18	0.36	0.01	0.02±0.01	1.59	1.06	1.78	3.75±0.54	
ALA	0.33		0.07	0.28			2.14	0.72	1.48	4.36±1.20	
ASN/SER	0.67	0.02		0.82		0.02	2.17	1.01	2.07	3.68±0.25	0.01
ASP	1.82		0.01	0.36	0.19		0.53	2.29	0.98	1.62±0.76	
CYS	0.17					0.46±0.07					0.12
GLN		0.09	3.56	0.7		0.02					0.03
GLU	0.9		0.09	0.37	0.02	0.02	2.39	1.55	2.63	9.23±1.52	
GLY									0.03	0.13±0.03	
PRO	0.26	1.59		1.35	1.31	1.75±0.67			1.08	1.97±0.20	0.65
TYR	0.14	0.01	0.58	0.16	0.01	0.14±0.09	0.45	0.25	0.43	0.61±0.05	0.12
Total	**8.34**	**1.82**	**6.92**	**5.93**	**1.64**	**2.81**	**13.84**	**9.49**	**14.72**	**32.02**	**1.85**
Sugar (%)	9.6	14.6	11.7	14.4	20.4	23.28±1.44	8.3	11.2	11.5	9.25±1.13	16.8

Table 1 (continued)

Species	*rupestris*		*speciosa*	*spicata*	*vanbalenii*	*vryheidensis*		*wickensii*	
	(1)	(2)			(*N*=4)	(1)	(2)	(1)	(2)
ARG				0.09	0.67±0.11	0.07	0.25		0.71
HIS									
ILE	0.24	0.51		0.7	0.02±0.01	0.42	0.54		
LEU	0.08	0.2		0.19	0.02±0.00	0.25	0.36		
LYS	0.05			0.24	0.16±0.03	0.18	0.64		
MET		0.29		0.28	0.05±0.00	0.59	0.61		0.06
PHE	0.39	0.8		0.66	0.13±0.05	0.36			
THR				0.27		0.47	0.97		
TRP	0.06	0.09		0.18	0.04±0.00	0.05	0.07		
VAL	0.44	1.24		1.36	0.05±0.02	1.45	1.86		
ALA				0.46	0.04±0.01	1.78	2.34		
ASN/SER	0.21	0.73		1.03	0.09±0.02	3.2	8.21	0.02	0.02
ASP	1.73	2.05	5.11	1.19	0.02±0.00	2.15	0.1	0.01	
CYS									0.21
GLN				0.71	0.18±0.05	1.38	3.08	0.01	0.03
GLU	0.93	1.37		0.59	0.10±0.01	0.52			0.01
GLY					0.01	0.2	0.29		
PRO	1.16	1.03	0.38	0.47	0.99±0.31	2.03	0.72	1.36	0.5
TYR	0.14	0.27		0.31	0.06±0.02	0.34	0.27		
Total	**5.43**	**8.58**	**5.49**	**8.73**	**2.19**	**15.44**	**20.31**	**1.4**	**1.54**
Sugar (%)	10.3	10	5.7	10	13.95±1.10	10.6	5.5	14.3	13.1

Amino acid concentrations in mM; sugar concentrations in % *w*/*w*; means±SE if four samples analyzed.

Table 2 Amino acids in the nectar of 10 *Erythrina* species

	Southern African Species						South American Species			
	lysistemon (*N*=7)	*caffra* (*N*=5)	*latissima*	*acanthocarpa*	*humeana*	*zeyheri*	*falcata*	*crista-galli*	*americana*	*flabelliformis*
ARG	0.66±0.18	0.64±0.12	1.71	0.47	0.47	0.01	0.85	0.98	0.07	0.10
HIS	0.37±0.12	0.24±0.08	0.65	0.56	0.42		0.15		0.05	0.10
ILE	1.06±0.12	0.85±0.23	1.14	0.35	0.94		0.35	1.44	0.04	0.24
LEU	1.35±0.15	1.32±0.31	1.46	0.46	0.90		0.15	0.55	0.04	0.24
LYS	0.45±0.27	0.38±0.05	0.70	0.30	0.86	0.01	0.72	2.02	0.03	0.14
MET	1.01±0.16	0.60±0.11	0.66	0.62	0.58	0.01	0.15	0.37		0.08
PHE	0.76±0.17	0.25±0.14	0.36	0.19	0.23		0.04	0.36	0.01	0.04
THR	0.89±0.26	1.22±0.41	1.03	0.29	0.38		0.25	1.82	0.04	0.13
TRP		0.03±0.01	0.03	0.09			0.01	0.06		0.01
VAL	1.97±0.21	1.37±0.37	2.28	0.99	0.98	0.12	0.70	2.24	0.18	
ALA	0.60±0.14	1.31±0.32	4.72	0.00	0.27	0.02	1.39	4.61		0.56
ASN/SER	23.05±3.28	24.29±7.39	31.04	7.21	6.43	0.47	6.84	26.06	3.57	4.65
ASP	0.34±0.23	2.46±2.46		0.79	0.07	0.08	0.09	0.51	0.16	0.25
CYS	0.01±0.00						0.02			
GLN	17.30±2.27	4.79±1.36	4.30	18.27	10.59		1.28	2.70	0.49	4.29
GLU	0.07±0.04	0.43±0.42	0.01	0.10		0.02	0.04	0.06	0.04	0.67
GLY	0.03±0.02	0.22±0.10		0.06	0.03					0.07
PRO	4.84±0.75	3.52±0.66	0.99	0.46	0.62	0.06	0.86	1.09	0.51	0.57
TYR	0.17±0.03	0.28±0.07	0.33	0.42	0.04		0.06	0.33		0.03
Total	**54.93**	**44.19**	**51.41**	**31.62**	**23.82**	**0.80**	**13.95**	**45.20**	**5.23**	**12.16**
Sugar (%)	17.1±0.8	10.1±1.0	10.6	11.2	14.2	20.5	16.3	21	32.5	34

Amino acid concentrations in mM; sugar concentrations in % *w*/*w*; means±SE for *E. lysistemon* and *E. caffra*.

Table 3 Amino acids in the nectar of *Schotia*, *Greyia*, *Cotyledon*, *Strelitzia*, and *Melianthus*

	Schotia brachypetala	*Greyia flanagani*		*G. sutherlandii*	*G. radlkoferi*	*Cotyledon orbiculata*	*C. barbeyi*		*Strelitzia reginae*		*S. nicolai*		*Melianthus major*	*M. comosus*
	(*N*=3)	(1)	(2)	(*N*=4)			(1)	(2)	(1)	(2)	(1)	(2)		
ARG	0.01									0.26			1.26	0.71
HIS	0.11													
ILE	0.13						0.01						0.15	0.07
LEU	0.07				0.02	0.01	0.02	0.02						
LYS	0.01								0.02		0.02			
MET	0.05±0.02					0.01						0.10	0.17	0.1
PHE	0.03						0.03	0.01						
THR	0.06												0.32	0.16
TRP							0.02	0.02						
VAL	0.17±0.01	0.05	0.12	0.07±0.01	0.11	0.12	0.02	0.01						
ALA	0.09±0.04						0.01	0.02						
ASN/SER	0.49±0.40						0.02	0.01	0.02				0.1	
ASP	0.08±0.03						0.04	0.04	0.01					
CYS													0.46	0.28
GLN	0.20±0.12						0.17	0.08	0.02					
GLU	0.06±0.02					0.01		0.02	0.01		0.01	0.01		
GLY	0.03													
PRO	2.73±1.64					0.02	1.47	1.32		0.97		0.75	0.83	0.52
TYR	0.04						0.09	0.01	1.31	0.78		0.12	0.25	
Total	**3.87**	**0.05**	**0.12**	**0.07**	**0.13**	**0.17**	**1.90**	**1.56**	**1.39**	**2.01**	**0.03**	**0.98**	**3.54**	**1.84**
Sugar (%)	19.3±3.2	11.4	11.2	8.4±0.7	10.0	20.7	21.6	21.6	20.4	18.5	5.6	8.8	17.8	19.1

Amino acid concentrations in mM; sugar concentrations in % *w*/*w*; means±SE if three or four samples analyzed

spaces in Tables 1, 2 and 3 to highlight the limited numbers of amino acids present in the nectars of some plant species. Note that in all tables data for essential amino acids are presented before data for nonessential amino acids.

Some *Erythrina* species had high total amino acid concentrations and a profile that was heavily skewed toward nonessential amino acids: asparagine/serine made a major contribution to the total concentration in *E. caffra*, *E. lysistemon*, *E. latissima*, and *E. crista-galli* (Table 3). Most essential amino acids were present in the nectar of *Erythrina* species, but at low concentrations. Apart from *E. caffra*, *E. lysistemon*, and *E. latissima*, another three southern African species were analyzed: *E. acanthocarpa* and *E. humeana* had high amino acid concentrations, but the value for *E. zeyheri* was low. For the four South American species, nectar amino acid concentrations were lower (and sugar concentrations higher) in the hummingbird-pollinated *E. americana* and *E. flabelliformis* than in *E. crista-galli* and *E. falcata* (Table 3).

In contrast to the *Aloe* and *Erythrina* species, amino acid concentrations were consistently low in the nectar of the other bird-pollinated genera investigated: *Greyia* (all three species of this genus were sampled), *Cotyledon*, *Strelitzia* (two of the three species of the genus), *Schotia*, and *Melianthus* (Table 4). The nectar of four plants of *Greyia sutherlandii* and two of *G. flanagani* contained a single amino acid, valine, at a mean concentration of <1 mM. The nectar of a single plant of *G. radlkoferi* contained only 0.11 mM valine and a trace of leucine (Table 4).

Effect of pollen on nectar amino acids Nectar sugar concentrations in the three *A. marlothii* plants used in the pollen contamination experiment were similar: 9.6%, 10.6%, and 13.7%. Total concentrations of about 50 mM amino acids in the nectar of these three plants

Table 4 Effect of pollen addition on nectar amino acid concentrations in *Aloe marlothii*

Amino Acid	Treatment		$F_{(1,2)}$		
	Control Nectar	Pollen Added	Treatment	Duplicate	Treatment × Duplicate
ILE	2.52±0.24	2.53±0.11	<0.01	0.72	0.02
LEU	1.93±1.00	1.88±0.95	0.11	2.24	4.00
LYS	0.50±0.06	0.50±0.05	0.03	1.10	0.21
MET	0.72±0.06	0.74±0.02	0.17	1.57	0.01
PHE	3.19±0.35	3.18±0.14	<0.01	0.44	0.02
THR	2.17±0.28	2.46±0.21	14.85	0.82	0.37
TRP	0.25±0.03	0.24±0.02	0.02	0.45	0.16
VAL	6.18±0.46	6.19±0.26	<0.01	0.25	0.02
ALA	4.05±0.36	4.20±0.22	0.48	0.89	0.06
ASN/SER	12.34±0.86	12.88±0.61	0.67	0.83	0.01
ASP	2.40±0.08	1.46±0.12	35.97	1.09	0.01
GLN	0.12±0.01	0.31±0.17	3.94	1.71	4.00
GLU	9.66±1.49	11.00±0.53	0.69	0.90	0.16
GLY	0.29±0.02	0.35±0.03	29.43	0.94	0.61
PRO	2.17±0.10	2.32±0.02	2.54	1.52	0.48
TYR	0.91±0.10	0.89±0.03	0.04	0.60	<0.01
Total	49.40	51.13			

Amino acid composition was analyzed in duplicate for three *A. marlothii* nectar samples (control nectar) and for the same three samples after addition of pollen (5 mg/ml) (pollen added). Amino acid concentrations in mM (mean±SE) with the results of 15 repeated measures ANOVA indicating the *F* values (*df*=1,2) for the different sources of variance (treatment, duplicate, treatment × duplicate).

Table 5 Variation among trees and inflorescences in the nectar amino acids of *Erythrina lysistemon*

	Tree (*df*=5)			Inflorescence (*df*=18)		Duplicate (*df*=24)	
Amino acid	*F*	%	*r*±SE	*F*	%	%	*r*±SE
ARG	3.75	(39.9)	0.41±0.24	28.15**	(56.0)	(4.1)	0.96±0.02
HIS	13.51**	(73.7)	0.76±0.14	8.77**	(20.9)	(5.4)	0.94±0.02
ILE	6.35*	(56.8)	0.57±0.21	59.14**	(41.8)	(1.4)	0.98±0.01
LEU	1.24	(5.6)	0.06±0.2	110.01**	(92.7)	(1.7)	0.98±0.01
LYS	3.52	(38.5)	0.39±0.24	127.04**	(60.6)	(1)	0.99±0
MET	0.51	(0)	−0.14±0.12	89.93**	(97.8)	(2.2)	0.98±0.01
PHE	1.27	(5.2)	0.06±0.2	4.1**	(57.6)	(37.1)	0.63±0.13
THR	8.42**	(62.7)	0.65±0.19	9.66**	(30.3)	(7)	0.92±0.03
VAL	5.39*	(52)	0.52±0.22	78.55**	(46.8)	(1.2)	0.99±0.01
ALA	11.20**	(71.3)	0.72±0.16	39.76**	(27.3)	(1.4)	0.98±0.01
ASN/SER	18.17**	(80.8)	0.81±0.12	47.54**	(18.4)	(0.8)	0.99±0
ASP	3.27	(36.2)	0.36±0.24	1,166.3**	(63.7)	(0.1)	1±0
GLN	6.80*	(58.1)	0.59±0.21	21.33**	(38.2)	(3.8)	0.96±0.02
PRO	4.74*	(48)	0.48±0.23	73.45**	(50.6)	(1.4)	0.99±0.01
TYR	1.37	(8.4)	0.08±0.21	1,146.87**	(91.5)	(0.2)	1±0

Variation in amino acid concentration was analyzed among six different *trees*, four different *inflorescences* per tree and between two different *duplicate* measurements per nectar sample. Nested ANOVA results (*F* values), the proportion of variance explained by each categorical variable (values in parenthesis; %) and repeatability±one standard error (*r*±*SE*) are presented. Significant values (following a Bonferroni correction) are indicated with asterisks. Significance at the 0.001 level could not be detected after Bonferroni correction because SAS only reports significant levels up to 0.0001 (0.0001/15=0.00007).

*$P<0.05$

**$P<0.01$

(Table 5) were higher than the mean value for four other *A. marlothii* plants from different locations (Table 1). Cysteine was absent. Glutamic acid and asparagine/serine were particularly high in these samples, but the addition of pollen had no effect on the amino acids (Table 5). Although an initial mildly significant effect of treatment was detected for ASP and GLY ($P=0.027$ and $P=0.032$, respectively), this did not remain significant for either amino acid following a Bonferroni correction.

Variability in nectar amino acid composition Nectar samples of *E. lysistemon* collected from six different trees showed high and fluctuating levels of glutamine and asparagine/serine, even among inflorescences on a single tree, and also high levels of proline (range 5.6 to 25.6 mM, mean 11.6 mM; data not shown). Total concentrations of amino acids in these nectar samples ranged from 50 to 150 mM with a mean±SE of 97.5±5.5 mM, which is much higher than the mean value for seven other *E. lysistemon* trees shown in Table 3. For the 24 samples from 6 trees used to analyze variability, the nectar sugar concentration measured with a refractometer ranged from 12.9% to 28.0% (mean 18.5%), and the total amino acid concentration was significantly correlated with the sugar concentration: $y=6.147x-16.396$ ($F_{(1,22)}=54.89$; $R^2=0.701$; $P<0.001$). This is to be expected, as both will vary with the water content of the nectar, but note that the amino acids also contribute to the refractometer readings (Inouye et al. 1980).

Because of their absence from more than two thirds of the 24 nectar samples, CYS, GLY, GLU, and TRP were excluded from the analysis. Even when present, these amino acids were barely detectable (e.g., TRP 0.06±0.03 mM; $N=7$) and are probably present in trace

Table 6 Variation among trees and inflorescences in the nectar amino acids (corrected for total concentration) of *Erythrina lysistemon*

	Tree (*df*=5)			Inflorescence (*df*=18)		Duplicate (*df*=24)	
Amino acid	*F*	%	*r*±SE	*F*	%	%	*r*±SE
ARG	1.3	(6.8)	0.07±0.21	39.77**	(88.6)	(4.6)	0.95±0.02
HIS	9.23**	(61.3)	0.67±0.18	3.36**	(20.9)	(17.7)	0.81±0.07
ILE	1.93	(18.8)	0.19±0.23	193.97**	(80.4)	(0.8)	0.99±0
LEU	0.45	(0)	−0.16±0.11	474.67**	(99.6)	(0.4)	1±0
LYS	3.46	(38)	0.38±0.24	832.92**	(61.8)	(0.1)	1±0
MET	2.31	(24.7)	0.25±0.24	710.27**	(75.1)	(0.2)	1±0
PHE	0.24	(0)	−0.23±0.07	9.24**	(80.5)	(19.5)	0.77±0.08
THR	13.42**	(71.7)	0.76±0.15	4.43**	(17.9)	(10.4)	0.88±0.05
VAL	2.82	(31.1)	0.31±0.24	247.27**	(68.3)	(0.6)	0.99±0
ALA	18.62**	(80.9)	0.81±0.12	25.78**	(17.7)	(1.4)	0.98±0.01
ASN/SER	9.15**	(66.9)	0.67±0.18	119.15**	(32.6)	(0.6)	0.99±0
ASP	4.48	(46.5)	0.47±0.23	947.32**	(53.4)	(0.1)	1±0
GLN	28.31**	(87.1)	0.87±0.08	136.24**	(12.7)	(0.2)	1±0
PRO	7.97**	(62.9)	0.64±0.19	33.26**	(35.0)	(2.2)	0.98±0.01
TYR	1.13	(3.2)	0.03±0.19	2,557.99**	(96.8)	(0.1)	1±0

Details as for Table 6, except that concentrations of individual amino acids were adjusted to a total amino acid concentration of 97.5 mM (the mean value for the 24 *E. lysistemon* inflorescences).

*$P<0.05$

**$P<0.01$

amounts only. The nested ANOVA analysis was carried out by using data from both uncorrected and corrected (i.e., absolute and relative) amino acid concentrations. In general, the between-inflorescence proportion of variance comprised most of the variance in nectar amino acid concentrations for both uncorrected (Table 5) and corrected concentrations (Table 6). Indeed, significant variation in the concentration among inflorescences was detected for all amino acids included in the analysis, whereas only a few showed significant between-tree variation (Tables 5 and 6). The between-tree variance for ILE and VAL was no longer significant when amino acid composition was corrected for total concentration. The proportion of variance explained by duplicate measures of the same nectar sample was low (Tables 5 and 6). Although an *F* statistic could not be computed for duplicate measures because of the lack of an error term, the high and significant repeatability of the duplicate measures suggests insignificant variation between them.

Discussion

Flowers pollinated by passerine birds generally have dilute nectars (Nicolson 2002), but some of these have surprisingly high amino acid concentrations. Inflorescences from the six trees used in examining variation in *E. lysistemon* had a mean total amino acid concentration of almost 100 mM, and the nectar of *A. marlothii* used in the pollen experiment had a total concentration of around 50 mM. In contrast, total amino acids in the nectar of 30 insect-pollinated species analyzed by Gardener and Gillman (2001a) ranged from 0.19 to 12.7 mM. Direct comparison with the data of Petanidou et al. (2006) is not possible because these authors expressed amino acids as picomoles per flower.

The distinctive ornithophilous genus *Erythrina* (Fabaceae) has a pantropical distribution and is unusual in including both hummingbird and passerine pollinators. Do my data for the nectar amino acids of *Erythrina* species support the clear pattern demonstrated by Baker and Baker (1982b), using ninhydrin staining read on a spectrophotometer, in relation to bird pollination in this genus? Three of the southern African species, *Erythrina lysistemon*, *E. caffra*, and *E. latissima*, have high amino acid concentrations and are pollinated by a variety of passerine birds, mainly unspecialized nectarivores (Jacot Guillarmod et al. 1979). In the other southern African species analyzed, concentrations vary from <1 mM in *E. zeyheri* to 32 mM in *E. acanthocarpa*. *Erythrina zeyheri* and *E. humeana* are pollinated by sunbirds (Jacot Guillarmod et al. 1979). Pollinators are not known for *E. acanthocarpa*, but sunbirds are likely candidates. Among the South American species, *Erythrina crista-galli* has high amino acid concentrations. Its basal position in the genus (Bruneau 1996) fits with the idea of passerine pollination and high nectar amino acids being ancestral, but field data show it is pollinated by bees and hummingbirds (Galetto et al. 2000). The correlation between nectar amino acid concentration and type of bird pollinator is thus not as strong as postulated by Baker and Baker (1982b), but *E. americana* and *E. flabelliformis* are clear examples of hummingbird-pollinated species with high nectar sugar concentrations and relatively low amino acids.

In nectars of the 16 *Aloe* species analyzed, total amino acid concentrations ranged from low in most species (<10 mM) to high in *A. marlothii* (Table 1). As in the passerine-pollinated species of *Erythrina* (Baker and Baker 1982b), *Aloe* nectar sugars are mainly glucose and fructose (van Wyk et al. 1993). In fact, hexose-dominated nectars are common to all the genera examined in this study (Nicolson 2002). In the case of a 10% pure hexose nectar with a combined concentration of 576 mM glucose and fructose, 50 mM amino acids (as in some individuals of *A. marlothii*) may make a substantial contribution to the refractive index, and this confirms the warning of Inouye et al. (1980) that some nectar sugar concentrations may be overestimated. However, the amino acid contribution to refractive index will be negligible in insect-pollinated flowers with more concentrated nectars and low amino acid concentrations (Gardener and Gillman 2001a).

There are many sources of variation in nectar amino acid composition and concentration. Some nonessential amino acids play a central role in the nitrogen metabolism of plants and make up a high proportion of the amino acids in phloem sap (Douglas 1993; Pate et al. 1985, 1998), so it is not surprising that they also dominate the amino acid profiles in many nectars. In *E. lysistemon*, glutamine, asparagine, and serine together comprised 64% of the total amino acid concentration in nectar of the six trees used to examine intraspecific variation. In nectar of *Impatiens capensis* (Balsaminaceae), glutamine and hydroxyproline, a component of plant cell walls, accounted for most of the intraspecific variability in nectar amino acids (Lanza et al. 1995). Proline comprises almost half the total amino acids in several species of *Nicotiana* (Solanaceae), but the concentrations are low (Kaczorowski et al. 2005). Whereas Carter et al. (2006) considered 2 mM proline in the nectar of ornamental tobacco to be high, proline concentrations averaged 11.6 mM in the 24 inflorescences of *E. lysistemon* used to assess intraspecific variation.

Some differences among studies are caused by differences in methodology. For example, Petanidou et al. (2006) were unable to identify proline or cysteine in the nectars of their large sample (73 species) of eastern Mediterranean plants. Phenylalanine was the dominant amino acid in these nectars, averaging 17% of the total and being especially high in Lamiaceae. Phenylalanine is considered important for bee pollinators in the Mediterranean region (Petanidou et al. 2006), because of its phagostimulatory effect in insects (Gardener and Gillman 2002), but is less evident in other published analyses. Tryptophan was absent from

the nectars of all but one of the 30 plant species analyzed by Gardener and Gillman (2001a), but present in most of those of Petanidou et al. (2006). Additional sources of variation in nectar amino acids include diurnal and seasonal fluctuations in the amino acid composition of phloem sap (Pate et al. 1998; Winter et al. 1992). Growth conditions are also important: Gardener and Gillman (2001b) showed that fertilizer treatment of *Agrostemma githago* (Caryophyllaceae) led to substantial increases in glutamine and proline. Large increases in one or two amino acids will change the relative abundance of all the rest, which is an argument for using absolute rather than relative concentrations. In other studies, Gardener and Gillman (2001a) found composition to be less variable than concentration, and Petanidou et al. (2006) used proportions for their statistical analyses. In *E. lysistemon*, most of the variance in nectar amino acids occurred among inflorescences regardless of whether absolute or relative concentrations were examined. Caution is needed in interpreting my results, as data for 21 out of 36 plant species are based on a single point analysis.

In this study, pollen falling into the nectar was not a source of variation in the nectar amino acids of *A. marlothii*. Confidence in statistical results based on a sample size of three is generally low. However, the use of duplicate analyses in assessing the effect of pollen on amino acid composition controlled for experimental error and, in general, the repeatability of the amino acid analyses for duplicate samples was exceptionally high (Tables 5 and 6). In *A. marlothii*, at least, the results are unlikely to be confounded by pollen contamination during nectar collection. This is in marked contrast to the findings of Gottsberger et al. (1990) who measured dramatic increases in nectar amino acids after pollen addition: 10 pollen grains of *Hibiscus rosa-sinensis* were sufficient to increase the amino acid content of 20 µl of nectar. Although proline remained low after pollen addition to the nectar of *A. marlothii*, it has been reported as the main amino acid released from immersed pollen (Gottsberger et al. 1990; O'Brien et al. 2003). The latter study was in connection with specialized pollen-feeding behavior in *Heliconius* butterflies, but the authors used maize pollen, which bursts when soaked in water or sugar solutions (Human and Nicolson 2003). Whether or not dislodged pollen adds amino acids to nectar may depend on the size and structure of the pollen grains.

There have been only two investigations of the behavioral responses of birds to the inclusion of amino acids in artificial nectar. Neither hummingbirds nor sunbirds selected sugar solutions containing amino acids at low concentrations, and both reduced their feeding rates at higher concentrations (Hainsworth and Wolf 1976; Leseigneur et al. 2007). Among the nonessential amino acids, sunbirds showed no significant avoidance of 15 mM glutamine in 20% sucrose, but did avoid asparagine, serine, and proline at the same concentration (Leseigneur et al. 2007). However, many of the plant species examined in this study produce large volumes of dilute nectar and are visited less by specialized sunbirds than by occasional nectarivores such as bulbuls, starlings, weavers, and mousebirds (Johnson and Nicolson, unpublished data). Moreover, unlike sunbirds, these occasional nectarivores are seemingly undeterred by the bitter taste of some nectars, which is caused by phenolic compounds in the black nectar of *A. vryheidensis* (Johnson et al. 2006) and by unknown compounds, possibly alkaloids, in *E. caffra*. Gardener and Gillman (2002) have drawn attention to the potential contribution of amino acids to the taste of nectar, and it seems likely that the taste of amino acids will be more apparent in dilute nectars. The combination of low sugar and high amino acid concentrations in nectars of some bird-pollinated flowers means that relatively large amounts of amino acids may be ingested in relation to the energy gained, with potential contributions to nitrogen balance of the birds.

Acknowledgments Many people gave permission to sample nectars, and I particularly want to thank the Pretoria National Botanic Garden, Andrew Hankey at the Walter Sisulu National Botanic Garden (Johannesburg), and Veronica Botha who has South American *Erythrina* species in her private garden. Amino acid analyses were carried out by Annette Exley, Department of Biochemistry, University of Pretoria. I am grateful to Braam van Wyk for botanical advice and Luke Verburgt for statistics. This research was supported by the South African National Research Foundation and the University of Pretoria.

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and tyrosol were tested in *in vitro* bioassays and showed strong dose-dependent inhibitory effects against *P. ramorum*, *P. cinnamomi*, *P. citricola*, and *P. citrophthora*. These results suggest that phloem chemistry varies in response to pathogen infection in California coast live oak populations and that changes in phloem chemistry may be related to apparently resistant phenotypes observed in the field.

Keywords Sudden oak death · Resistance · *Quercus agrifolia* · Canker · *In vitro* bioassay

Introduction

Phytophthora ramorum Werres, de Cock, and Man in't Veld, the causal agent of sudden oak death (Werres et al. 2001; Rizzo et al. 2002), was first observed killing tanoaks (*Lithocarpus densiflorus*) in Marin County, California in the mid 1990s (Svihra 1999). Since then, the pathogen has been found in 14 coastal counties in California and 1 southwestern county in Oregon. To date, the pathogen has been responsible for widespread mortality of tanoaks, as well as several oak species, including coast live oak (*Quercus agrifolia*), California black oak (*Q. kelloggii*), and Shreve oak (*Q. parvula* var Shrevei) (Garbelotto et al. 2001). The pathogen is known to occur in North America and Europe, and has a wide host range of over 40 plant genera (Rizzo et al. 2005). In the US, the impact of the disease on tanoaks and coast live oaks has been particularly significant with *P. ramorum* in some sites killing 100% of the mature tanoaks and 45% of coast live oaks (McPherson et al. 2005). At such sites, the death of these trees is having a dramatic ecological impact as these species provide food and habitat for animals, serve as fire breaks, and host mycorrhizal networks (Pavlik et al. 1991; Davidson et al. 2005). The destruction of keystone oak species could ultimately lead to reduced biodiversity in these ecosystems and changes in forest composition (Rizzo and Garbelotto 2003).

In oaks and tanoaks, the primary observable symptom of infection is the development of stem cankers that produce a deep red exudate from intact bark (Garbelotto et al. 2001; Rizzo et al. 2002; McPherson et al. 2005). The canker is caused by the pathogen infecting phloem tissue, and frequently extends into the outer portions of the xylem (Rizzo et al. 2002; Brown and Brasier 2007). A black line is usually evident at the margins of the infected areas of the phloem and xylem (Davidson et al. 2003). Other symptoms include brown and white boring dust on the surface of the bark and/or moss caused by ambrosia and bark beetles tunneling into the bleeding cankers (McPherson et al. 2005).

The apparent resistance to *P. ramorum* infection of some individuals within coast live oak populations has been observed in artificial inoculation studies (Dodd et al. 2005; McPherson et al., unpublished data). For example, from artificial branch-cutting inoculation trials, Dodd et al. (2005) found variation (up to eightfold difference in lesion sizes) in susceptibility to *P. ramorum*. In addition, apparent resistance has also been observed in naturally infected forests where a small proportion of infected coast live oaks have survived for extended periods in populations with high mortality levels (McPherson et al. 2005).

Elevated levels of secondary metabolites, specifically phenolic compounds in infected tissue, are often associated with resistance to fungal pathogens in angiosperms (Ostrofsky et al. 1984; Bennett and Wallsgrove 1994). It is possible that these apparently resistant coast live oaks may have increased amounts of phenolic compounds in the *P. ramorum* infected tissue, which are inhibiting the growth of the pathogen. However, no data are available showing changes in secondary metabolites of coast live oaks infected with *P. ramorum*. To date, the majority of studies investigating phenolic chemistry in oak have focused on constitutive wood

and foliage chemistry (e.g., Mammela et al. 2000; Salminen et al. 2004; De Simon et al. 2006).

P. ramorum is a newly discovered pathogen (see review by Rizzo et al. 2005), and there is a need for knowledge concerning the biology of the disease, including a better understanding of the defense responses of oaks infected with *P. ramorum*. We investigated the chemical defense response of California coast live oaks to *P. ramorum* infection by comparing concentrations of the soluble phenolics extracted from healthy and infected trees, and we tested the inhibitory activity of differentially expressed phenolics against pathogenic *Phytophthora* species (including *P. ramorum*) in *in vitro* bioassays.

Methods and Materials

Phloem was sampled from artificially inoculated, naturally infected, and healthy coast live oaks at three different times, December 2004, May 2005, and September 2005 at field sites located in Deer Island, Open Space Preserve (38° 6′ N, 122° 32′ W) and China Camp State Park, Marin County (38° 0′ N, 122° 30′ W), California.

The main goals in the following experiments were to induce cankering (or detect natural cankers) and study the associated host response to the necrogenic activity of the pathogen. Therefore, reisolations of the pathogens were not attempted. Previous studies have shown that *P. ramorum* is the only *Phytophthora* species detected in the cankers produced by using the same inoculation technique and samples cultured from natural cankers found in Marin County (N=44) (McPherson, unpublished data). Furthermore, the appearance of cankers resulting from our inoculations was entirely consistent with the appearance of natural cankers.

The phloem lesions (i.e., the cankers) resulting from artificial inoculations in experiments 1 and 3 covered vertical distances ranging from a few centimeters to over 50 cm (unpublished data). However, exact canker sizes were not measured because, on large stems, the cankers are extremely irregular and the pathogen appears to weave in and out of the phloem (Brown and Brasier 2007). Consequently, cankers are often a collection of smaller lesions with no well-defined edge to the infection, which makes it difficult to accurately measure lesion size.

Set-up for experiment 1: comparison between artificially inoculated and apparently healthy trees Coast live oaks that did not display bleeding cankers or irregular crowns, i.e., that looked healthy, were randomly selected at Deer Island. The diameter at breast height (i.e., approximately 1.5 m above ground) of the selected trees ranged from approximately 28 to 69 cm. The GPS coordinates were recorded for one tree, and the rest of the trees were located in this general area. The trees were then randomly assigned to no treatment (control) and treatment with *P. ramorum*. Each treatment and control consisted of five independent experimental units (trees), for a total of ten trees.

On June 29, 2004, five randomly selected trees were inoculated with *P. ramorum* isolate FOP 10-9-1 with three inoculations per tree at breast height by using a modification of the procedure described by Rizzo et al. (2002). A hole (2 cm diameter) saw was used to remove plugs of bark and phloem tissue down to the sapwood. Five-millimeter agar plugs colonized with an actively growing culture of *P. ramorum* on V-8 agar were placed against the sapwood, and the bark plugs were replaced. Aluminum foil was placed over the wounds, and plugs were held in place with duct tape to minimize contamination and desiccation of cut tissue.

On December 13, 2004, the outer bark over and around the inoculation sites was removed with a hand axe. Samples of inner phloem from the margins of the cankers were harvested, placed in plastic bags, and stored at ambient temperature in the field. Samples of

inner phloem were harvested in the same way from corresponding positions on the trunk of healthy trees. All samples were stored at 4°C upon returning to the laboratory and until extracted.

Set-up for experiment 2: comparison between naturally infected and apparently healthy trees Seven naturally infected trees and five apparently healthy asymptomatic trees were randomly selected for sampling in May 2005 at the China Camp State Park. Trees putatively infected with *P. ramorum* were selected based on symptoms, i.e., the presence of exudate from a canker. Using the same harvesting method described above, phloem was sampled from the active margins of the cankers as well as asymptomatic phloem located at least 60 cm above the cankers on each infected tree (these samples will be henceforth referred to as AFC samples for "away from the canker"). Phloem was sampled from five trees showing no symptoms of *P. ramorum* infection. Samples were placed on dry ice in the field and stored at 4°C upon returning to the laboratory and until extracted.

Set-up for experiment 3: comparison between cankered and noncankered phloem within trees The five uninoculated control trees that were sampled in experiment 1 at Deer Island were used in experiment 3. They had been wounded for sampling on December 13, 2004. These five trees were inoculated with *P. ramorum* (isolate from Jack London State Park, Glen Ellen, California) on June 30, 2005 in areas of the trunk that had not been wounded previously. Phloem samples were collected on September 12, 2005 from canker margins and AFC positions as described for experiment 2. Samples were placed on dry ice in the field, then transported to the laboratory, and stored at 4°C upon returning to the laboratory and until extracted.

Chemical analyses Phloem tissue was frozen in liquid nitrogen and then ground to a powder with a mortar and pestle. Soluble phenolics were extracted with 1 ml of high-performance liquid chromatography (HPLC) grade methanol (Fisher) from 100 mg FW ground phloem tissue for 24 h at 4°C. Particulate matter was removed by centrifugation (5 min at 12,000×*g*). Soluble phenolics were analyzed by HPLC on an Alliance 2690 separation module equipped with an autosampler and a 996 model photo diode array detector (Waters, Milford, MA, USA). A Waters Xterra RP18, 5 μm, 4.6×150 mm column was used. With the exception of a different acidified water–methanol solvents system, the same operating conditions as detailed by Bonello and Blodgett (2003) were performed. Here, 2% formic acid was replaced with 2% acetic acid, and no bases were added to either water or methanol. The photo diode array detector was set to scan between 237 and 400 nm (Bonello and Blodgett 2003), and simultaneous monitoring was performed at 280 nm (flavan-3-ols) and 370 nm (bicyclic coumarin derivatives).

Peak identification and quantification To identify peaks that showed a treatment-dependent behavior, samples within treatments were pooled by combining 50 μl from each sample. The pooled samples were analyzed by HPLC to provide a representative chromatogram for each treatment (Bonello and Blodgett 2003). Nine of these peaks (Table 1) were then analyzed for all individual samples.

UV spectra and retention times of the individual peaks were compared to the following standards: benzoic acid, caffeic acid, catechin, ellagic acid, 4-ethylguaiacol, 4-ethylphenol, gallic acid, ferulic acid, 4-methoxy-4-propylphenol, vanillin, vanillic acid, catechin, taxifolin, tyrosol (Sigma Aldrich, St. Louis, MO, USA), *trans*-4-cinnamic acid, *trans*-4-coumaric acid,

Table 1 Summary of nine phenolic compounds identified in the crude extracts of coastal live oak phloem

Identity (Abbreviated Name, Where Indicated)	RT (min)	Detector Channel for Quantification (nm)
Gallic acid	4.7	280
Tyrosol	7.0	280
Tyrosol-like (TY1)	8.7	280
Catechin	9.5	280
Ellagic acid derivative (EA1)	18.4	370
Ellagic acid derivative (EA2)	19.2	370
Ellagic acid	19.4	370
Ellagic acid derivative (EA3)	21.2	370
Ellagic acid derivative (EA4)	22.6	370

pinosylvin, pinosylvin monomethyl ether (Apin Chemicals, Abingdon, UK), peltatoside, and hyperoside (Extrasynthese, Genay, France).

Gallic acid, tyrosol, catechin, and ellagic acid (see Fig. 1 for structures) were unequivocally confirmed by spiking samples with the respective standards and comparing retention times and UV spectra. These four compounds were quantified by using a four-point regression curve (in a range of 0.005 to 5 mg/ml for catechin, gallic acid, tyrosol, and 0.001 to 1 mg/ml for ellagic acid) constructed with the available standards. Calibration curves with r^2 of 0.99 or better were used. One compound with RT of 8.7 min was identified as a tyrosol derivative based on its UV spectrum, which was similar to that of tyrosol. Similarly, four compounds with RT of 18.5, 19.2, 21.2, and 22.6 min were identified as ellagic acid derivatives based on their UV spectra, which were similar to that of ellagic acid. These compounds, henceforth referred to as TY1, EA1, EA2, EA3, and EA4, respectively, were quantified in terms of tyrosol and ellagic acid equivalents with UV detection at 280 nm for tyrosol and 370 nm for ellagic acid (see Table 1 for a summary of the nine compounds identified and quantified by HPLC).

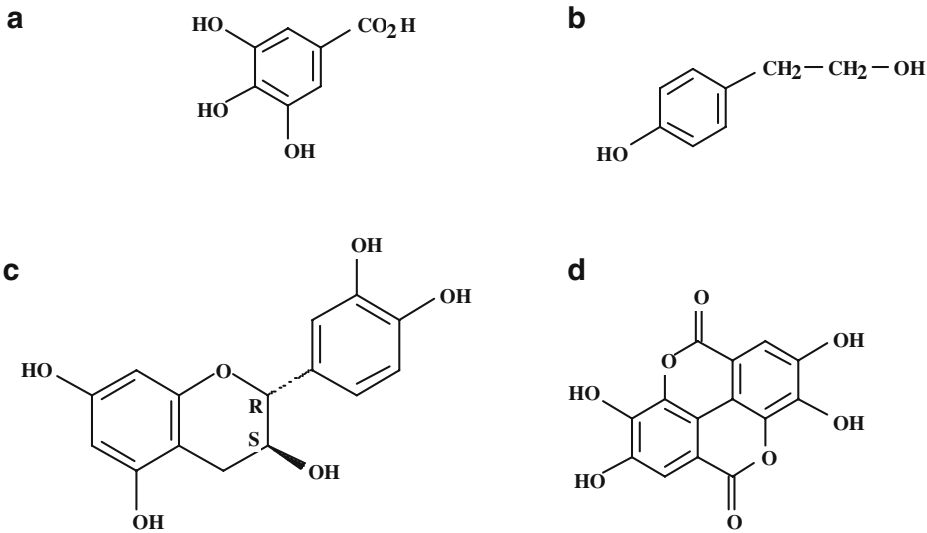


Fig. 1 Structures of the compounds identified in phloem extracts. **a** Gallic acid, **b** tyrosol, **c** catechin, **d** ellagic acid

In vitro bioassay The antifungal activities of gallic acid (2, 3, 6, and 10 mg/ml) and tyrosol (1, 2, 3, and 6 mg/ml) were tested against *P. ramorum*, *P. cinnamomi*, *P. citricola*, and *P. citrophthora in vitro*. Solubility problems prevented us from testing the activity of ellagic acid under the conditions of our assays. For each compound, the concentrations incorporated into dilute frozen lima bean agar (LBA) (Schmitthenner and Bhat 1994) (using the equivalency of 1 ml medium=1 g fresh weight of phloem) were based on the range of concentrations found in the crude extract across all experiments and treatments, i.e., gallic acid, 0.0–5.63 mg/g FW; tyrosol, 0.0–3.25 mg/g FW. In practice, we selected a range of concentrations including approximately twice the highest concentration found in the tissues. This was based on the rationale that individual tissue measurements resulted from the homogenization of tissue that included both necrotic and nonnecrotic areas at the canker margins. Thus, compound concentrations in phloem cells located at the pathogen's invasion front may be underestimated by the "mean" concentration found in the tissue sample analyzed.

Gallic acid changed the medium pH to 3.9, so a corresponding control of LBA, pH 3.9, was used (medium acidified with HCl). Tyrosol did not change the medium pH, so the control for this treatment was unamended LBA (pH=6.7). The same unamended LBA was used as a baseline control in the gallic acid treatment (Fig. 2). In each case, a 3-mm plug of actively growing mycelium was placed onto the center of LBA plate, and mycelial growth (colony diameter) was measured after 4 days incubation at room temperature in the dark. Three replicate Petri dishes/species/chemical/concentration were used. The bioassays with *P. ramorum* were conducted in the laboratory of Dr. David Rizzo at UC Davis.

Statistical analyses For experiment 1, the data on the effect of infection status on concentrations of the identified compounds were analyzed by using multivariate ANOVA. For experiment 2, the data on the effects of infection status and sampling position within trees (canker or AFC, for the infected trees only) on compound concentrations were analyzed with ANOVA by using an unbalanced split plot model with infection status as the whole-plot and sampling position as the subplot. For experiment 3, our data on the effect of sampling position (canker or AFC) on compound concentrations were analyzed with a paired *t*-test. Data from the bioassay were analyzed with univariate ANOVA with compound/concentration and species used as factors. Fisher's least significant difference (LSD) was used to compare the means in all tests. In all analyses, Levene's test was used to examine variance equality, and log or square root transformations were used to eliminate or minimize variance heterogeneity. All tests were conducted using SPSS 11.0 for Windows or MacOS X.

Results

Quantification of Phenolic Compounds

Experiment 1: comparison between artificially inoculated and apparently healthy trees Infected phloem contained greater amounts of gallic acid (20-fold) and ellagic acid (64-fold) than healthy phloem ($F_{1,9}$=32.491, P<0.001; $F_{1,9}$=8.312, P<0.03, respectively). In contrast, infected phloem contained 22% of the catechin and 7% of the EA2, present in healthy phloem ($F_{1,9}$=24.893, P<0.002; $F_{1,9}$=17.048, P<0.002, respectively). Infection status did not affect the concentrations of tyrosol, TY1, EA1, EA3 or EA4 (Table 2).

Experiment 2: comparison between naturally infected and apparently healthy trees As in experiment 1, the concentration of gallic acid was higher in the cankers of infected trees than in

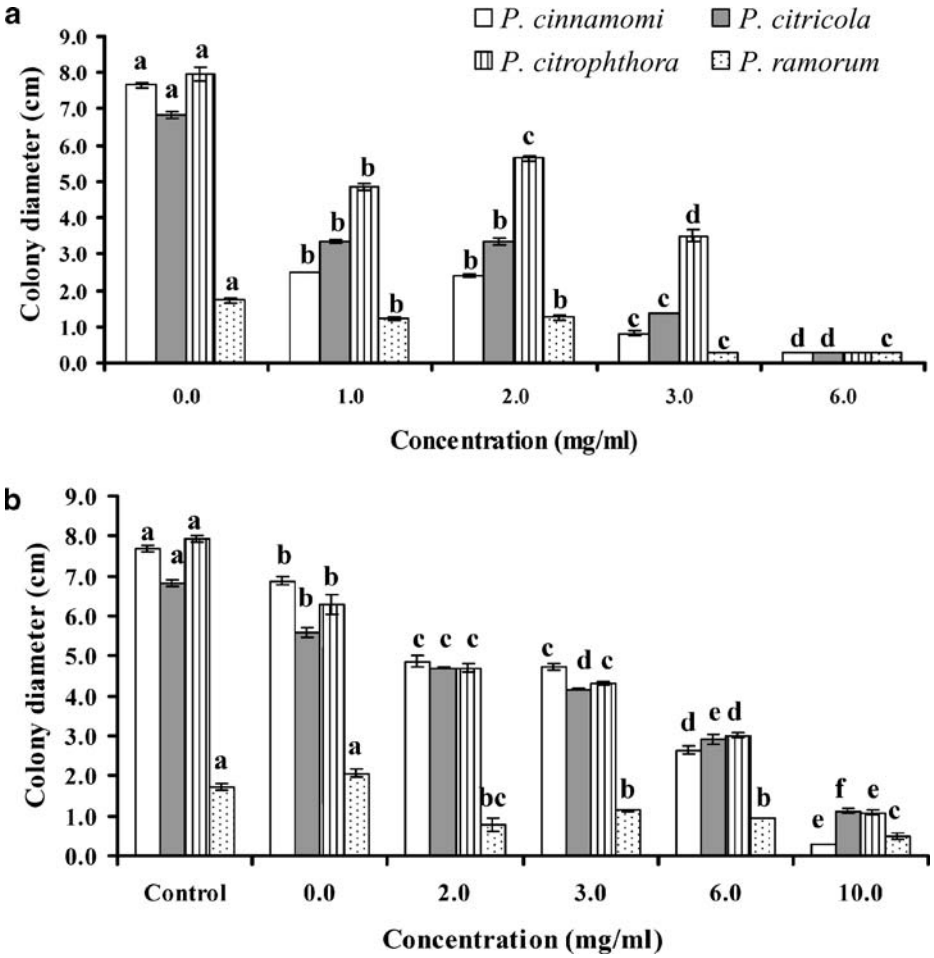


Fig. 2 Effects of tyrosol (**a**) and gallic acid (**b**) on colony growth rates for *P. cinnamomi*, *P. citricola*, *P. citrophthora*, and *P. ramorum*. Each bar represents the mean of three replicates ±1 SEM and includes the diameter of the original inoculum plug (3 mm). Different letters indicate significant differences ($P<0.05$, univariate ANOVA, LSD test) within species only. Species was a significant factor in all ANOVAs, indicating that the four *Phytophthora* species are affected differentially by both treatments. The control for tyrosol (0.0 mg/ml) was used as a baseline and is reproduced as the control bars in **b**

either the phloem of asymptomatic trees (13-fold) ($F_{1,15.9}=28.34$, $P<0.001$) or AFC phloem (10-fold) ($F_{1,8.3}=36.74$, $P<0.001$). AFC and asymptomatic phloem did not differ significantly in gallic acid levels ($F_{1,15.9}=0.01$, $P>0.93$) (Table 3). Ellagic acid was present in greater amounts (4-fold) in cankers of infected trees than in AFC phloem ($F_{1,9.1}=6.14$, $P<0.05$).A trend of greater levels of ellagic acid in infected phloem compared with healthy phloem was also detected ($F_{1,13.7}=3.57$, $P=0.08$). The difference between AFC and healthy phloem was not significant ($F_{1,13.7}=0.84$, $P>0.37$). The concentrations of tyrosol, TY1, catechin, EA1, EA2, EA3, and EA4 were not different among infected, AFC, and healthy phloem (Table 3).

Experiment 3: comparison between cankered and noncankered phloem within trees Tyrosol, catechin, and ellagic acid were found in different amounts in infected and AFC phloem

Table 2 Effect of artificial *P. ramorum* inoculation on the concentration of nine phenolic compounds extracted from the phloem of coast live oak in experiment 1

	Experimental Factors[a]	
Compound	Infected (N=5)	Healthy (N=5)
Gallic acid	1.97 (0.93) a	0.09 (0.03) b
Tyrosol	0.91 (0.35)	1.01 (0.18)
TY1[b]	2.88 (0.77)	4.08 (0.69)
Catechin	0.63 (0.16) a	2.87 (0.35) b
EA1[c]	0.11 (0.05)	0.39 (0.16)
EA2[c]	0.03 (0.01) a	0.37 (0.17) b
Ellagic acid	1.47 (0.69) a	0.02 (0.01) b
EA3[c]	0.11 (0.06)	0.35 (0.09)
EA4[c]	0.09 (0.06)	0.10 (0.02)

Values in each row followed by different letters are significantly different (P<0.05); multivariate ANOVA, LSD test.

[a] All concentrations expressed as mg/g fresh weight (SE).

[b] Compound quantified in terms of tyrosol equivalents.

[c] Compounds quantified in terms of ellagic acid equivalents.

collected in May 2005. Infected phloem had higher concentrations of tyrosol (3-fold) and ellagic acid (14-fold) than AFC tissue (t_4=2.86, P<0.05; t_4=6.84, P<0.01, respectively), whereas catechin was present in infected tissue at 31% of the amount found in AFC phloem (t_4=3.09, P<0.05) (Table 4). No differences were found between infected and AFC samples for the following compounds: gallic acid, TY1, EA1, EA2, EA3, and EA4 (Table 4).

In vitro bioassay Results of the bioassay are summarized in Fig. 2. All treatments had significant effects on colony growth rates for all four *Phytophthora* species. Tyrosol

Table 3 Effect of natural *P. ramorum* infection on the concentration of nine phenolic compounds extracted from the phloem of coast live oak in experiment 2

	Experimental Factors[a]		
Compound	Infected (N=7)	AFC (N=7)	Healthy (N=5)
Gallic acid	0.94 (0.29) a	0.09 (0.02) b	0.07 (0.01) b
Tyrosol	0.82 (0.25)	1.13 (0.20)	1.26 (0.17)
TY1[b]	2.89 (0.56)	2.71 (0.34)	2.58 (0.23)
Catechin	3.52 (0.59)	3.32 (0.54)	2.07 (0.19)
EA1[c]	0.36 (0.13)	0.43 (0.15)	0.32 (0.06)
EA2[c]	0.15 (0.05)	0.31 (0.16)	0.14 (0.05)
Ellagic acid	0.16 (0.05) a	0.04 (0.02) b	0.04 (0.02) ab
EA3[c]	0.21 (0.10)	0.20 (0.12)	0.10 (0.10)
EA4[c]	0.13 (0.06)	0.15 (0.07)	0.09 (0.08)

Values in each row followed by different letters are significantly different (P<0.05), ANOVA, LSD test.

AFC: asymptomatic phloem located at least 60 cm above the canker.

[a] All concentrations expressed as mg/g fresh weight (SE).

[b] Compound quantified in terms of tyrosol equivalents.

[c] Compounds quantified in terms of ellagic acid equivalents.

Table 4 Effect of artificial *P. ramorum* inoculation on the concentration of nine phenolic compounds extracted from the phloem of coast live oak in experiment 3

	Experimental Factors[a]	
Compound	Infected	AFC
Gallic acid	0.42 (0.30)	0.47 (0.21)
Tyrosol	2.23 (0.33) a	0.75 (0.57) b
TY1[b]	2.19 (0.87)	4.25 (1.32)
Catechin	1.18 (0.34) a	3.84 (0.88) b
EA1[c]	0.21 (0.13)	0.40 (0.16)
EA2[c]	0.18 (0.12)	0.37 (0.18)
Ellagic acid	0.53 (0.16) a	0.04 (0.01) b
EA3[c]	0.07 (0.03)	0.23 (0.10)
EA4[c]	0.14 (0.06)	0.12 (0.05)

Values in each row followed by different letters are significantly different ($P<0.05$), paired *t*-test.

AFC: asymptomatic phloem located at least 60 cm above the canker.

[a] All concentrations expressed as mg/g fresh weight (SE).

[b] Compound quantified in terms of tyrosol equivalents.

[c] Compounds quantified in terms of ellagic acid equivalents.

completely inhibited growth of all four species at the highest concentration, but significant effects could be observed at lower concentrations. *P. citrophthora* appeared to be the least sensitive of the four species to tyrosol. An apparently linear dose response for all four species was observed with gallic acid, although medium acidity also appeared to be a factor (albeit minor) in slowing growth rates for *P. cinnamomi*, *P. citricola*, and *P. citrophthora*, but not *P. ramorum*.

Discussion

Increased concentrations of secondary compounds, especially phenolic compounds, have been reported to occur in the lesion margins of many woody tree species and are generally considered to be implicated in protection against pests and diseases (Hart and Hillis 1974; Woodward and Pearce 1988; Evensen et al. 2000; Viiri et al. 2001, Eyles et al. 2003; Bonello and Blodgett 2003; Blodgett et al. 2007). In the present study, significant differences in phenolic profiles were demonstrated between phloem sampled from the active margins of cankers, healthy phloem from asymptomatic trees, and phloem sampled 60 cm above canker sites in coast live oaks. In particular, five compounds (gallic acid, tyrosol, catechin, ellagic acid, and EA2) were present in differing concentrations in the various treatments. Given that these compounds were present in the healthy phloem as well as in the infected phloem, they are considered constitutive compounds, in contrast to *de novo* produced compounds that are synthesized only in response to a pathogen attack and that would only be detected in infected tissue. This result confirms the lack of evidence, to our knowledge, for phenolics produced *de novo* in response to pathogen attack in oaks.

It should be noted that the production of phenolic compounds was not consistent across all experiments. Specifically, greater amounts of ellagic acid and gallic acid were observed in infected phloem than noninfected phloem in experiments 1 and 2. In contrast, higher amounts of tyrosol and ellagic acid were present in infected phloem than corresponding controls in

experiment 3. It is interesting to note that catechin levels were reduced in infected tissue in two of the three experiments, i.e., experiments 1 and 3. In general, the artificially inoculated trees accumulated more phenolics in response to the infection than the naturally infected trees. We suggest that the artificially inoculated trees, which had been more recently infected with an active pathogen, stimulated a more active host response. As we were unable to determine when the naturally infected trees were inoculated, we cannot speculate on the effect that timing of inoculation has on phenolic production and accumulation. Furthermore, it is possible that other factors such as seasonality (i.e., phenological status) or even the method used to store tissue (room temperature vs dry ice) might have contributed to the observed variability. These factors should be investigated further. The use of controlled branch inoculations to correlate elevated phenolic levels with certain infection characteristics including canker size and host resistance (e.g., Blodgett et al. 2007) may also be helpful.

The soluble phenolic compounds identified in the phloem extracts of infected coast live oak have been implicated as playing key roles in the defense against fungi and herbivores in many woody species including other *Quercus* spp. (Malterud et al. 1985; Pearce 1996; Feucht and Treutter 1999). For example, the durability of some hardwood species against microbes has been directly attributed to the elevated presence of hydrolyzable tannins (Klumpers et al. 1994; Hillis 1999; Barry et al. 2001; Vivas et al. 2004). Hydrolyzable tannins refer to both ellagitannins and gallotannins. The former are esters of hexahydroxydiphenoyl groups with a sugar core and often contain galloyl groups. In contrast, gallotannins consist of a sugar substituted only with galloyl groups (Okuda et al. 1995; Barry et al. 2001).

Hart and Hillis (1972) conducted bioassays with white oak (*Quercus alba* L.) heartwood extracts containing ellagitannins and observed inhibition of the growth of *Poria monticola* Murrill, a wood decay fungus. The capacity of tannins to inhibit the growth of microbes has been credited to the ability of tannins to bind to proteins (Field and Lettinga 1992; Kawamoto et al. 1997) and their antioxidant activity (Okamura et al. 1993; Hagerman et al. 1998). In another study, tyrosol, catechin, and oleuropein were among the main phenolic compounds identified in the stem cortex of olive trees (Del Rio et al. 2003). The stem cortex of olive tree is commonly attacked by a *Phytophthora* sp. causing rot and necrosis, and Del Rio et al. (2003) showed that of the three compounds, tyrosol was the most active growth inhibitor of *Phytophthora* in an *in vitro* bioassay. Of particular note, the greatest antifungal effect was achieved with all three compounds combined. The authors suggested that the increased accumulation of phenolic compounds in the cortex was a defense mechanism of olive plants against the pathogen. Our bioassay results confirmed the toxicity of tyrosol against *Phytophthora* spp., including *P. ramorum*, and indicate that gallic acid also has antimicrobial activity. It is possible that both compounds (and others we have not tested) may have synergistic activities.

In summary, this study demonstrated clear host secondary metabolite responses that may be implicated in the resistance of coast live oak to attack by *P. ramorum*. Further studies involving correlation of compound concentrations with disease resistance *in planta* will be necessary to establish a potential defensive role for any of these compounds. If such a role is established, then some of these compounds could be used as biomarkers in the selection of resistant coast live oak genotypes. These studies, however, are contingent on developing techniques to obtain quantitative measures of host resistance (e.g., based on lesion size as in Blodgett et al. 2007), which are lacking at present in a form that is conducive to concurrent characterization of defense mechanisms.

Acknowledgments The authors would like to thank Nadir Erbilgin, Tom Gordon, Gabriela Ritok-Owens, and Pavel Svihra for the assistance in the field and the lab, David Rizzo for providing the *P. ramorum*

isolates, and Matthew Dileo for conducting the bioassay with *P. ramorum*. Special thanks to Larry Madden for the assistance with the statistical analyses and Duan Wang for conducting the fungal bioassays with *P. cinnamomi*, *P. citricola*, and *P. citrophthora*. Access to research sites was provided by Marin County Open Space District and China Camp State Park. Salaries and research support were provided by state funds appropriated to the Ohio Agricultural Research and Development Center, the Ohio State University, and the U.S. Forest Service (04-CA-11244225-432).

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test materials. Few differences in time budgets were observed among aphids exposed to individual components, but synthetic blends and trapped headspace volatiles from PLRV-infected plants resulted in significantly less time spent walking by aphids than synthetic blends and trapped headspace from non-infected controls. Our results indicate that arrestment of *M. persicae* by PLRV-infected plants requires the blend of volatile organic compounds released by these plants and is not produced in response to a single compound.

Keywords Volatile organic compounds · Blends · Electroantennogram · Plant viruses · Vectors · Potatoes · Green peach aphid

Introduction

Despite early work that seemed to demonstrate otherwise (Kennedy et al. 1959a, b), olfactory cues can influence aphid responses to host plants (Petterson 1970, 1973; Chapman et al. 1981; Campbell et al. 1990; Nottingham et al. 1991; Pickett et al. 1992; Nottingham and Hardie 1993; Hardie et al. 1995; Petterson et al. 1994; van Giessen et al. 1994; Visser and Piron 1997; Park and Hardie 1998; Quiroz and Niemeyer 1998; Eigenbrode et al. 2002; Jiménez-Martínez et al. 2004). In addition, aphids respond to plant volatiles both in field-trapping experiments and in laboratory olfactometer bioassays (Petterson 1970, 1973; Chapman et al. 1981; Nottingham et al. 1991; Petterson et al. 1994; Quiroz and Niemeyer 1998).

Volatiles released by plants are altered by pathogen infection (Chaudry et al. 1998; Cardoza et al. 2002; Jiménez-Martínez et al. 2004; Huang et al. 2005; McLeod et al. 2005), but the adaptive significance of these changes is obscure. Recent evidence indicates aphids are capable of responding behaviorally to differences in olfactory cues from plants infected with viruses and non-infected control plants (Eigenbrode et al. 2002; Jiménez-Martínez et al. 2004; Srinivasan et al. 2006). The aphid species in these studies are the principal virus vectors, and each aphid species exhibits improved performance on the infected plants, suggesting the patterns are part of co-adaptation between virus and the aphid on which it depends for transmission.

The green peach aphid, *Myzus persicae* (Sulzer) is the principal vector of *Potato leafroll virus* (PLRV; genus Polerovirus; Luteoviridae; Syller 1996). The aphid acquires the virus in a persistent manner, and PLRV particles are associated with the salivary glands of the aphid after acquisition (Harrison 1984). The aphid develops faster, has higher fecundity, and preferentially settles on cultivated potato, *Solanum tuberosum* L., infected with PLRV than on non-infected potato plants (Castle and Berger 1993; Castle et al. 1998). Eigenbrode et al. (2002) showed that preferential colonization of potatoes infected by PLRV by *M. persicae* is influenced by volatile emissions from PLRV-infected plants. They reported that potato plants infected with PLRV produced volatiles that attracted and arrested *M. persicae*. In choice tests with and without contact with the leaf surface, and conducted in darkness, more aphids were located on or near leaflets from PLRV-infected plants than on or near leaflets from plants infected with *Potato virus Y* (PVY), *Potato virus X* (PVX), non-infected controls, or sham-inoculated plants (exposed to virus-free aphids). Volatile organic compounds (VOC) trapped from the plant headspace of PLRV-infected potato plants were more attractive than headspace VOC trapped from healthy plants in a dual-choice bioassay. Similar results were reported by Jiménez-Martínez et al. (2004), studying the response of *Rhopalosiphum padi*, the bird cherry-oat aphid, to wheat plants infected by *Barley yellow dwarf virus* compared with non-infected control plants. More recently, Srinivasan et al.

(2006) showed that *M. persicae* performs better on PLRV-infected hairy nightshade *Solanum sarrachoides* (Sendtner) plants than on non-infected controls, and that both winged and non-winged forms of the aphid show a preference for virus-infected plants.

The specific mechanisms that influence aphid responses to volatile compounds from virus-infected plants have not been investigated. It is possible that one or several of the components elevated by virus infection are specific arrestants for the aphids. Alternatively, the entire blend released by the infected plants may be responsible. The situation is similar to that for changes in plant volatile release following arthropod herbivory (De Moraes et al. 1998; Du et al. 1998; Dicke 1999; Paré et al. 1999; Turlings and Fritzsche 1999; Kessler and Baldwin 2001). Only a few of these studies have examined the importance of, and attempted to isolate, the blends and individual blend components released by herbivore-attacked plants on the responding carnivores. The parasitoid *Cotesia marginiventris* is attracted to a synthetic mixture of 11 major components of the blend emitted by corn plants infested by the parasitoid host, *Spodoptera exigua*. The predatory mite *Phytoseiulus persimilis* is attracted to four chemicals identified in the blend emitted by Lima bean plants infested with their prey, the spider mite *Tetranychus urticaethus* (Dicke et al. 1990).

Eigenbrode et al. (2002) reported that at least 11 headspace volatile components (*cis*-2-hexen-1-ol, *n*-nonane, nonanal, undecane, β-myrcene, (*R*)-(+)-limonene, (−)-*trans*-caryophyllene, (+)-longifolene, α-pinene, β-pinene, and α-humulene) were elevated by PLRV infection but not by PVX or PVY infection or sham inoculation compared with non-infected plants. In this study, we follow up on the report of Eigenbrode et al. (2002) to determine if individual VOC or blends of these compounds that are elevated as a result of PLRV infection elicit significant behavioral and physiological activity in the green peach aphid. Our specific objectives were to: (1) determine arrestment of the green peach aphid to blends of VOC elevated as a result of PLRV infection and those that are produced by non-infected potato plants, (2) determine arrestment of green peach aphid by individual, pure VOC elevated as result of PLRV infection, (3) determine the electrophysiological (EAG studies) response of *M. persicae* to individual VOC compounds, and (4) compare aphid movement rates and time allocated to specific behaviors when exposed to pure individual VOC or blends of these compounds elevated as a result of PLRV infection.

Methods and Materials

Insects The virus-free aphid colony originated from the "OUR" clone (maintained by T. Mowry at the University of Idaho Research and Extension Center in Parma, Idaho) and was maintained on seedlings of *Physalis floridana* Rybd. We tested second- to third-instar apterous aphids from this colony.

Test Compounds *Cis*-2-hexen-1-ol, *n*-nonane, nonanal, undecane, β-myrcene, (*R*)-(+)-limonene, (−)-*trans*-caryophyllene, (+)-longifolene, α-pinene, β-pinene, and α-humulene were purchased from Aldrich (St. Louis, MO, USA). All these VOC were elevated at least twofold without overlapping standard errors about the mean based on trapped headspace of five infected and five healthy plants (Eigenbrode et al. 2002). They are compounds from the fatty acid/lipoxygenase pathway (*cis*-2-hexen-1-ol), 9 and 10-carbon aliphatics (*n*-nonane, nonanal, undecane), monoterpenes ((*R*)-(+)-limonene, β-myrcene, α-pinene, β-pinene), and sesquiterpenes ((−)-*trans*-caryophyllene, (+)-longifolene, α-humulene). Serial dilutions of each compound in paraffin oil (Sigma Chemical Company, St Louis, MO, USA) were prepared to make all doses tested. Compounds were blended in ratios that mimicked ratios

at which they are present in the headspace of PLRV-infected and non-infected potato plants. Various concentrations were tested depending on the experiment.

Doses Each compound was dissolved in paraffin oil to obtain 1,000 mg/ml solutions. Dilutions were done until the desired concentrations were achieved. For the electroantennography (EAG) studies (see below), an intermediate dose of 5×10^{-5} ng per paper leaf model was tested. Individual chemical compounds in emigration bioassays were tested at doses of 4×10^{-7}, 2×10^{-6}, 1×10^{-5}, 5×10^{-5}, and 2.5×10^{-4} ng per paper leaf model. In direct observation studies, a dose of 2.5×10^{-4} ng per paper leaf model was used. Synthetic blends were tested at either 2.5×10^{-4} ng per paper leaf model (representing the concentration present in the headspace of PLRV-infected plants) or 1.25×10^{-4} (representing the concentration present in the headspace of non-infected plants). The synthetic blends were prepared by adding each component at a concentration that approximated that found in natural headspace collections and dissolved in paraffin oil, making a total blend concentration of 100 mg/ml. Dilutions were done until the desired concentrations were achieved. Natural headspace volatiles were also tested at 2.5×10^{-4} ng per paper leaf model (PLRV-infected) and 1.25×10^{-4} (non-infected plants).

Electroantennography Physiological activity of *M. persicae* when exposed to the compounds implicated in aphid responses to infected plants was assessed with electroantennography. EAG was carried out by using a commercially obtained electoantennography system (Syntech, The Netherlands). Each compound was dissolved in paraffin oil to make 1% (*v*/*v*) solution. One hundred microliters of the solution was applied onto an 8× 60 mm strip of Whatman No. 2 filter paper, which was then inserted into a glass Pasteur pipette that was oriented above the antennae of the aphid. The blank control consisted of a strip of filter paper treated only with 100 μl of paraffin oil. The aphid's antennae were attached across electrodes under a microscope. An air stimulus controller (model CS-05b, Syntech) was used for the air and odor delivery. A constant flow (10 ml/sec) of charcoal-filtered and humidified air passed over the antenna through the open end of the glass positioned 15 mm from the antenna. For most experimental procedures, a 2-min interval was maintained between simulations. Serial dilutions of the chemical compounds in paraffin oil were prepared to make an intermediate dose of 5×10^{-5} ng per paper leaf model. The maximum EAG amplitude was measured, the solvent blank response was subtracted, and the results were normalized to the response to linalool. Tests were replicated 10 times. Millivolt potentials for each chemical were averaged and compared to linalool, and the air control and each chemical response was also compared proportionally to linalool.

Behavioral Bioassays

Arrestment Arrestment of the green peach aphid to individual components, to synthetic blends of these components, and to a naturally occurring blend was measured with an emigration rate bioassay. This bioassay measures the rate at which aphids move away from an odor source, and was used previously to detect differential aphid emigration from virus-infected and healthy potato plants (Eigenbrode et al. 2002; Srinivasan et al. 2006). An arena made of a polystyrene 150-mm diam Petri dish was fitted with a false floor of polyethylene screening (mesh size ca. 1 mm) on which aphids could walk freely. One hundred microliters of the test solution were applied onto a filter paper (Whatman No. 2) cut to the approximate size of a potato leaflet (35 mm diam×55 mm length). The solution

was allowed to soak into the filter paper for 2 min and to begin releasing the dissolved test substance before introducing the aphids into the arena. Thirty apterous green peach aphids were placed directly above the paper leaf model on the mesh floor, and the arena was covered. Thereafter, aphid emigration was recorded every 10 min for 50 min. Aphids that left the leaf outline were considered emigrants and were removed and counted at each observation. Controls consisted of only filter paper in a leaf outline. Tests were replicated five times. Aphids were used once and then discarded. Prior bioassays of this nature had been carried out in darkness to avoid visual cues associated with infected vs. healthy plant tissue. The bioassays in the present study were conducted under normal laboratory lighting because all tested materials were visually equivalent. For the individual compounds tested, emigration rates in response to treatments were compared by using analysis of variance (ANOVA) followed by a least significant difference (LSD) means separation procedure (PROC GLM) in SAS (2002–2003). As for the synthetic blends, each treatment was fitted to the model $y = m*(1-\exp(-b*\text{time}))$, where y is the proportion emigrating, m is the maximum emigration, and b is the rate of emigration and time is in minutes. Following model estimation, parameter comparisons were done using full model dummy variable techniques.

Movement Rates and Specific Activities Behavior of individual aphids exposed to test compounds or blends was monitored and recorded to examine the basis of differences in emigration rates. One hundred microliters of the test compound were applied onto Whatman filter paper No. 2 beneath the floor of the arena and allowed to soak in and begin releasing the test compound for 2 min. Afterward, an individual aphid was placed onto the floor of the arena and observed continuously for 5 min. To observe the aphids and record their behavior, a tracking system developed for recording and quantifying animal behavior on artificial or natural substrates was used (Eigenbrode et al. 1989). It consists of an Apple Macintosh computer, a Summagraphics SummaSketch tablet (Summagraphics Corp., Fairfield CT, USA), a boom-mounted dissecting microscope equipped with an ocular crosshair and a mechanical stage manipulator salvaged from a compound microscope. We used this system for the analysis of the behavior of individual aphids when exposed to test compounds or blends. The behaviors of interest were: (1) walk, antenna forward; (2) stop, antenna back; (3) probe taken to describe short stopping periods when the aphid would be seen trying to place its stylet in the walking mesh; (4) walk, antenna back; (5) stop, antennae forward; (6) honeydew taken to describe periods when the aphid would stop and drop honey dew; (7) walk, antennae alternate; and (8) off the arena. Sixty aphids were observed for each of the treatments, and the observation time for each replicate was 300 sec. Wilk's lambda multivariate ANOVA (MANOVA) statistic assessed the significance of treatment effect on all behavioral categories simultaneously, and total time and proportion of time spent in each activity in response to the treatment was compared by using ANOVA followed by a least significant difference (LSD) means separation procedure (PROC GLM) in SAS (2002–2003).

Results

Electroantennography Among the compounds tested, only *cis*-2-hexen-1-ol, α-humulene, (−)-*trans*-caryophyllene, decanal, heptanal, (*R*)-(+)-limonene, and β-myrcene, elicited EAG responses from the aphid's antennae. *Cis*-2-hexen-1-ol and α-humulene elicited the highest

activities, whereas the pinenes and *n*-nonane elicited EAG responses that were lower than the control (Fig. 1).

Behavioral Bioassays

Arrestment

Emigration Rate Bioassays with Individual Compounds Among the VOC elevated as a result of PLRV infection, β-Pinene was a mild arrestant at concentrations of 4×10^{-7}, 2×10^{-6}, and 1×10^{-5} ng per paper leaf model (Table 1). The other compounds did not show any significant differences in the rate of emigration by green peach aphid at the different concentrations (4×10^{-7}, 2×10^{-6}, 1×10^{-5}, 5×10^{-5}, and 2.5×10^{-4} ng per paper leaf model) tested (Table 1).

Emigration Rate Bioassays with Synthetic Blends The synthetic blend that mimicked PLRV infection, and including all the compounds tested individually, was more arrestant to the aphids than either the synthetic blend that mimicked non-infected plants or a blank control (paraffin oil) (Fig. 2). For instance, after the first 10 min, a mean of 16 aphids had emigrated from PLRV mimic synthetic blends paper models compared to a mean of 24 aphids emigrating from paper leaf models treated with non-infected blend of plants (Fig. 2). When emigration data were fitted to the emigration rate model, significant differences were recorded both in the emigration rate (*b*) parameter and the maximum emigration rate (*m*) parameter (Table 2).

Emigration Rate Bioassays with Headspace Volatiles Emigration results with headspace volatiles collected from PLRV-infected and non-infected potato plants showed that green peach aphid is arrested by the PLRV-infected headspace volatiles blend, with fewer aphids emigrating from above paper leaf models treated with the PLRV-infected headspace volatiles compared to aphids emigrating from paper leaf models treated with non-infected headspace volatiles or a blank control (Fig. 3). When emigration data were fitted to the emigration rate model, significant differences were recorded both in the emigration rate (*b*) parameter and the maximum emigration rate (*m*) parameter (Table 3).

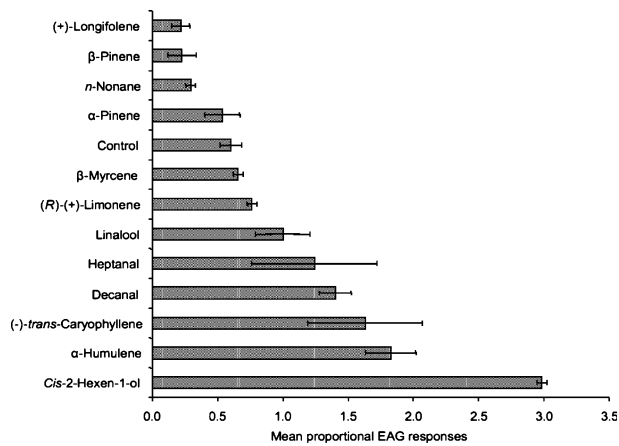


Fig. 1 Mean±SEM proportional mV EAG responses by *M. persicae* to selected VOC elevated by PLRV infection (responses standardized to linalool=1 and control = paraffin oil)

Table 1 Mean cumulative proportion of aphids emigrating when exposed to selected individual volatile organic compounds (VOC) elevated as a result of PLRV infection tested at five concentrations bracketing those present in the headspace VOC of PLRV-infected potato plants

Compound	Concentration Tested (ng Per Paper Leaf Model)				
	4×10^{-7}	2×10^{-6}	1×10^{-5}	5×10^{-5}	2.5×10^{-4}
Cis-2-hexen-1-ol	0.68 a	0.68 a	0.76 a	0.65 a	0.60 a
(−)-*trans*-caryophyllene	0.76 a	0.73 a	0.72 a	0.78 a	0.67 a
Decanal	0.74 a	0.79 a	0.72 a	0.76 a	0.70 a
Heptanal	0.66 a	0.65 a	0.61 a	0.62 a	0.56 a
α-Humulene	0.73 a	0.77 a	0.75 a	0.80 a	0.78 a
(*R*)-(+)-Limonene	0.72 a	0.65 a	0.66 a	0.71 a	0.67 a
(+)-Longifolene	0.66 a	0.67 a	0.69 a	0.71 a	0.72 a
β-Myrcene	0.69 a	0.69 a	0.69 a	0.68 a	0.66 a
α-Pinene	0.66 a	0.63 a	0.58 a	0.60 a	0.61 a
β-Pinene	0.63 b	0.64 b	0.60 b	0.68 ab	0.74 a
n-Nonane	0.64 a	0.71 a	0.72 a	0.74 a	0.72 a
Control	0.69 a	0.69 a	0.69 a	0.69 a	0.69 a

Numbers followed by the same letter within a row are not significantly different at the $P=0.05$ level (protected LSD).

Movement Rates and Specific Activities with Individual Compounds The data for the mean percent time spent by observed aphids in the various activities with each of the tested compounds are summarized in Table 4. Significant differences were observed for walking (which includes walking with antenna forward, walking with antenna behind, and walking antenna alternate; $F=1.88$; $P=0.039$), stopping (which includes stopping with antenna behind, stopping with antenna forward, and stopping with antenna alternate: $F=1.80$; $P=$

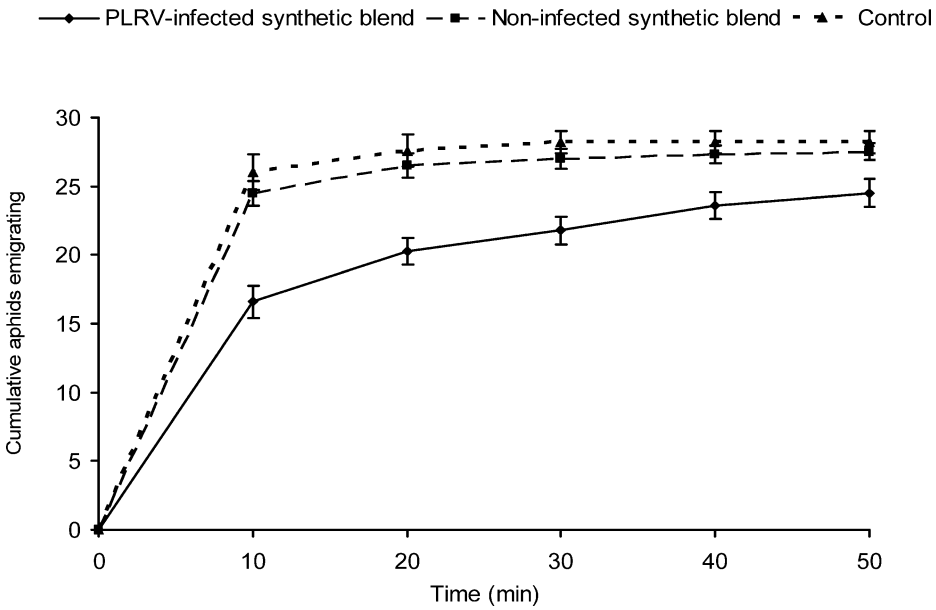


Fig. 2 Cumulative number of aphids emigrating from both infected and non-infected synthetic blends

Table 2 Emigration rates (BETA) and maximum emigration rates from both infected and non-infected synthetic blends tested at concentrations of 2.5×10^{-4} and 1.25×10^{-4} ng per paper leaf model

Treatment	Emigration Rate[a]	Maximum Emigration[b]	Contrasts (*P* value)[c]		
			Infected Blend	Non-infected Blend	Control
Infected blend	0.11±0.01	23.66±0.60	–	<0.001	<0.001
Non-infected blend	0.23±0.04	27.17±0.45	<0.001	–	0.248
Control	0.26±0.06	28.11±0.62	<0.001	0.248	–

[a] Emigration rate parameter (vector of slopes, *b*) from the equation $P = m\times[1 - \exp(-b\times\text{time})]$. Mean ± SEM for 30 aphids per treatment at the end of the 50-min bioassay observation period

[b] Maximum emigration parameter (*m*) from the equation $P = m\times[1-\exp(-b\times\text{time})]$. Mean ± SEM for 30 aphids per treatment at the end of the 50-min bioassay observation period

[c] Significance values from contrasts comparing regression curves performed using the equation $P = m\times[1-\exp(-b\times\text{time})]$

0.050), and probing (F=2.00; P=0.026). The category designated "other" (which includes off the arena, and producing honey dew) did not show significant differences (F=1.64; P= 0.083; Fig. 4), MANOVA statistics (Wilk's lambda, F value, 1.54 number *df*, 228, $P<$ 0.001). In the walking category, aphids spent more time walking when exposed to the control, followed closely by β-myrcene, nonanal, undecane, (*R*)-(+)-limonene, and *cis*-2-hexen-1-ol. Aphids also spent less time stopping when exposed to the control. Aphids stopped more when over the pinenes, followed closely by (*R*)-(+)-limonene, (−)-*trans*-caryophyllene, nonanal, and *n*-nonane. In the probing category, significantly more aphids

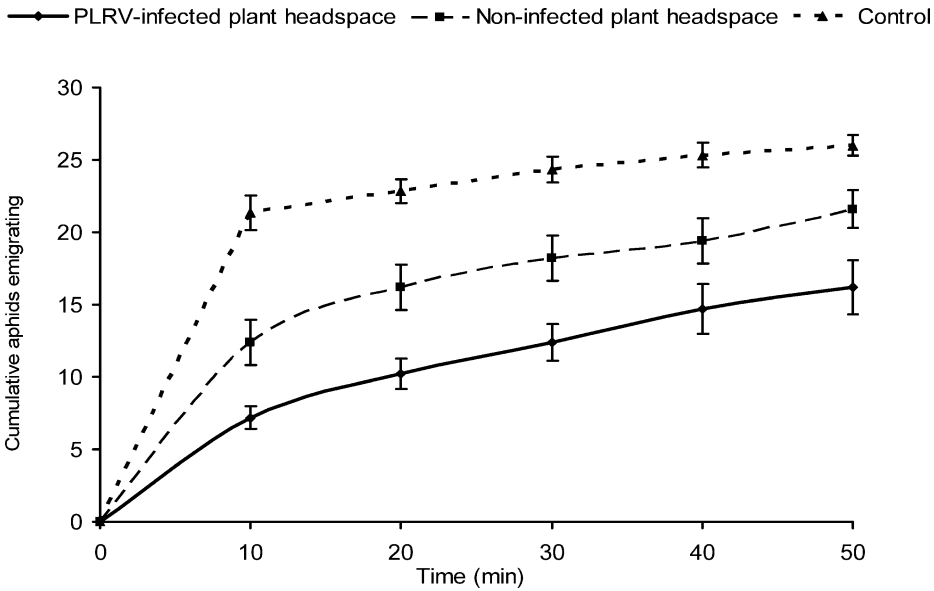


Fig. 3 Cumulative number of aphids emigrating from both PLRV-infected and non-infected plant headspace volatiles

Table 3 Emigration rates and maximum emigration rates from both PLRV-infected plant headspace and non-infected plant headspace volatiles tested at concentrations of 2.5×10^{-4} and 1.25×10^{-4} ng per paper leaf model

Treatment	Emigration Rate[a]	Maximum Emigration[b]	Contrasts (*P* value)[c]		
			Infected	Non-infected	Control
Infected	0.04±0.02	17.68±2.74	–	0.001	<0.001
Non-infected	0.08±0.02	20.78±1.29	0.001	–	0.004
Control	0.14±0.03	22.59±0.89	<0.001	0.004	–

[a] Emigration rate parameter (vector of slopes, *b*) from the equation $P = m\times[1-\exp(-b\times\text{time})]$. Mean ± SEM for 30 aphids per treatment at the end of the 50-min bioassay observation period

[b] Maximum emigration parameter (*m*) from the equation $P = m\times[1-\exp(-b\times\text{time})]$. Mean ± SEM for 30 aphids per treatment at the end of the 50-min bioassay observation period

[c] Significance values from contrasts comparing regression curves performed using the equation $P = m\times[1-\exp(-b\times\text{time})]$.

spent their time probing when over *cis*-2-hexen-1-ol compared to all other compounds including the control (Table 4, Fig. 4).

Movement Rates and Specific Activities with Synthetic and Pure Headspace Blends of PLRV-Infected and Non-Infected Potato Plants Aphids, exposed to both a synthetic blend made to mimic headspace from PLRV-infected plants at approximately natural headspace concentrations of 2.5×10^{-4} ng per paper leaf model and the headspace blends collected from PLRV-infected potato plants, spent more time stopping and less time walking than

Table 4 Mean percent (±SEM) time spent in various behavioral activities by *M. Persicae* when exposed to selected individual volatile organic compounds elevated as a result of PLRV infection

Compound	Behavior or Activity*			
	Walking	Stopping	Probing	Other*
Cis-2-hexen-1-ol	67.9±3.4 ab	21.3±2.9 bc	4.8±0.9 a	6.0±2.4 ab
α-Humulene	62.0±4.2 bc	31.9±4.1 a	2.1±0.4 bc	3.9±1.9 b
(−)-*trans*-caryophyllene	64.5±3.7 bc	29.4±3.6 ab	3.1±0.6 abc	3.0±0.6 b
Undecane	68.8±3.2 ab	27.3±3.3 ab	2.4±0.6 bc	1.5±0.3 b
n-Nonane	64.6±3.9 bc	29.3±3.7 ab	2.1±0.5 bc	4.0±1.7 b
(*R*)-(+)-Limonene	67.9±3.5 ab	24.4±3.3 abc	3.3±1.1 ab	4.5±1.7 b
β-Myrcene	69.2±3.5 ab	25.3±3.4 abc	3.2±0.9 abc	2.3±0.4 b
Control	76.3±2.7 a	17.0±2.4 c	1.8±0.7 bc	4.9±1.7 ab
α-Pinene	55.8±4.3 c	31.9±3.9 a	2.7±0.8 bc	9.6±3.6 a
β-Pinene	63.2±3.8 bc	31.9±3.7 a	1.5±0.4 bc	3.4±1.7 b
(+)-Longifolene	64.2±3.7 bc	30.8±3.7 ab	1.8±0.5 bc	3.2±1.7 b
Nonanal	68.2±3.5 ab	29.2±3.5 ab	1.4±0.3 c	1.2±0.1 b
ANOVA *F*, *df*	1.88, 11	1.80, 11	2.00, 11	1.64, 11
ANOVA *P* value	0.039	0.050	0.026	0.083

*Walking includes walking antenna forward, walking antenna behind, and walking antenna alternate; stopping includes stopping antenna forward, stopping antenna behind, and stopping antenna alternate; Other includes producing honey dew, and off the arena.

Numbers followed by the same letter within a column are not significantly different at the $P=0.05$ level (protected LSD).

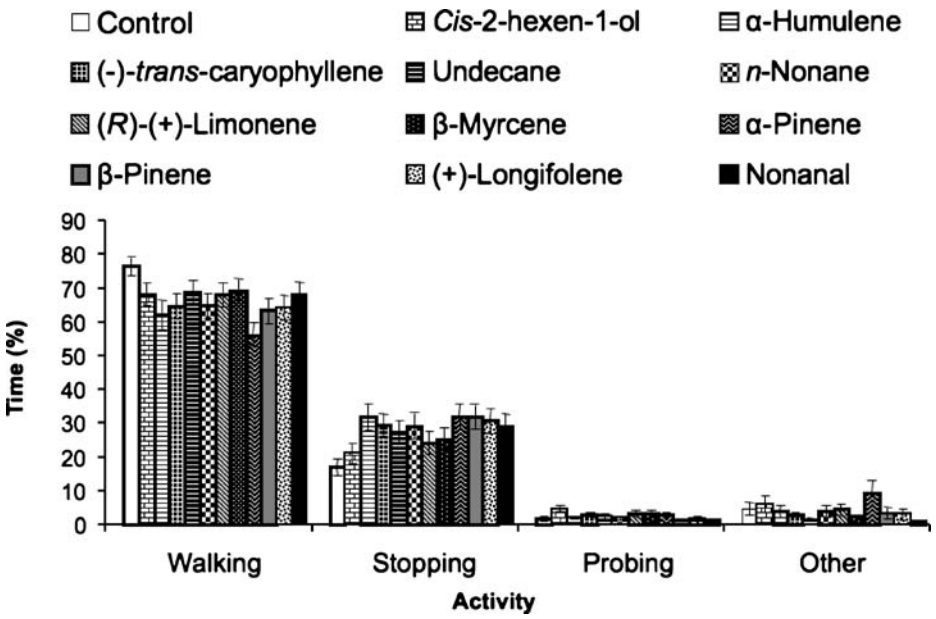


Fig. 4 Mean (±SEM) percent time *M. persicae* spent walking, stopping, probing, and other (which includes producing honey dew and off the arena) when exposed to individual synthetic volatile organic compounds elevated as a result of PLRV infection

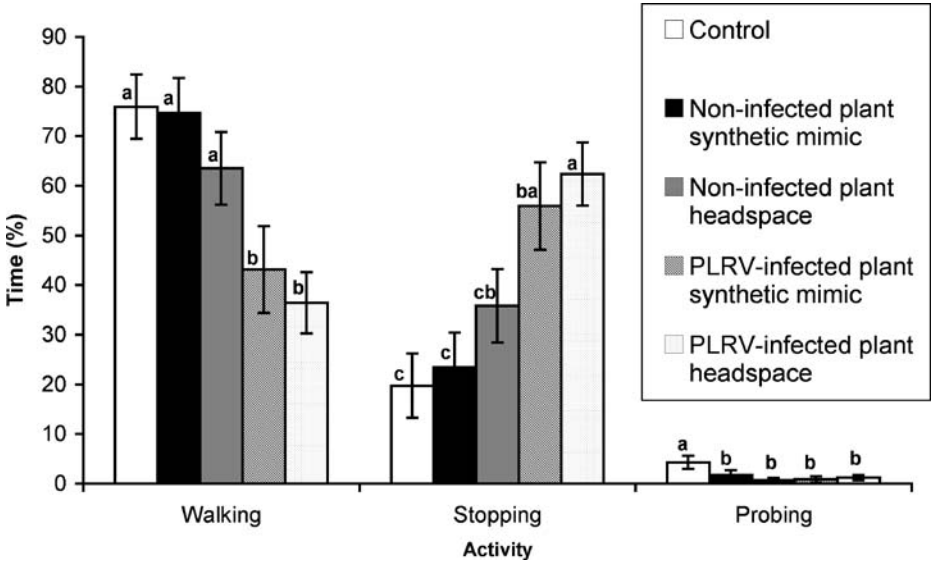


Fig. 5 Mean (±SEM) percent time *M. persicae* spent walking, stopping, and probing when exposed to synthetic and pure headspace blends of PLRV-infected and non-infected potato plants. *Bars* with the same letter are not significantly different at the $P=0.05$ level (protected LSD)

Table 5 Mean walking rate (±SEM) of *Myzus persicae* when exposed to headspace trapped from PLRV-infected and non-infected (sham-inoculated) potato plants and to synthetic blends of volatile organic compounds mimicking concentrations of those elevated as a result of PLRV infection

Treatment	Walking Rate (Mean±SEM)
Control (no plant)	10.03±1.65 a
Non-infected plant headspace	10.01±1.26 a
Non-infected plant synthetic mimic	7.73±0.93 a
PLRV-infected plant headspace	3.67±0.67 b
PLRV-infected plant synthetic mimic	4.26±1.05 b
ANOVA *F*	6.90
ANOVA *P* value	<0.001

Numbers followed by the same letter within a column are not significantly different at the $P=0.05$ level (protected LSD).

those exposed to a synthetic blend that mimicked non-infected potato plants (walking: $F=6.34$, $P=0.001$; stopping: $F=6.92$, $P<0.001$; probing: $F=3.08$, $P=0.019$), or those exposed to headspace blends collected from non-infected potato plants (Fig. 5). The aphids walked more slowly when exposed to either a synthetic blend made to mimic PLRV infection or pure headspace blend collected from PLRV-infected potato plants compared to either a synthetic blend made to mimic non-infected potato plants or pure headspace blends collected from non-infected potato plants ($F=6.90$, $P<0.001$) (Table 5).

Discussion

The study demonstrates that apterae of green peach aphid, the principal vector of PLRV (Syller 1996), respond to PLRV-infected potato plants preferentially compared to non-infected plants, and that this preference is influenced by volatile emissions from the PLRV-infected plants. These results are consistent with previous findings (Castle et al. 1998; Eigenbrode et al. 2002). Our results show further that a blend of volatiles produced by PLRV-infected plants, which differs in total concentration and relative composition from a blend from non-infected potato plants, is an arrestant for green peach aphid, whereas the individual components of the blend are not arrestants. To the best of our knowledge, this is the first study to demonstrate the importance of blends for *M. persicae* and the first to show the importance of blends vs. individual volatile compounds for eliciting behavioral responses in any aphid species.

In our EAG bioassays, results showed that some of the compounds elevated as a result of PLRV infection were perceived by the aphid's antenna (Fig. 1). There were some differences, however, in the intensity of response to particular compounds. For example, *cis*-2-hexen-1-ol, which is a green leaf volatile (GLV), and α-humulene, which elicits higher activity, whereas the pinenes and *n*-nonane caused EAG responses that were lower than the control. In prior studies, GLVs have elicited activity in EAG to other aphid species and green peach aphid (Hardie et al. 1995; Visser et al. 1996). Similarly, EAG responses to plant volatiles have been reported in several aphid species (Visser and Piron 1995; Visser and Yan 1995; Visser et al. 1996), and there are significant differences between EAG responses among the different aphid species (Hardie and Park 1998).

Most of the behavioral bioassays that investigate the responses of phytophagous insects to host volatiles usually report activity for blends of volatiles rather than for single compounds. For example, Fraser et al. (2003) used a blend of α-terpinene, methyl salicylate, (*E*)-ocimene, benzyl alcohol, decanal, nonanol, phenylacetaldehyde, and geranyl acetone at the same concentration as emitted by tomato foliage to attract *Manducta sexta* in a wind tunnel. They suggested that no single compound serves as a signature compound for host plant selection by the insect. In our system, observed arrestment of the green peach aphid onto PLRV-infected plants evidently requires the blend of VOC released by these plants and is not produced in response to a single compound. In our bioassays that tested individually several compounds elevated as a result of PLRV infection only β-pinene was mildly arrestant (Table 1), whereas none of the other individual compounds were especially active at the tested dose range (Table 1). However, the synthetic blend that mimicked headspace from PLRV-infected plants, and consisted of all compounds tested individually, was arrestant to the aphids compared with a synthetic blend that mimicked non-infected plants or a no-plant control. Response by aphids to these synthetic blends was similar to the response to intact trapped headspace blends from PLRV-infected and non-infected plants. The greater response of green peach aphid to blends of pure compounds than to individual compounds indicates a synergistic mechanism, similar to that observed for semiochemicals in other systems including pheromones, floral scents, and host volatiles (Silverstein and Young 1976; Linn et al. 1986; McLeod et al. 2005). The importance of blends and the sensitivity of insects to specific ratios have also been demonstrated in studies of parasitoid and predator responses to arthropod-feeding-induced release of VOC from plants (Dicke et al. 1990; Turlings et al. 1991).

Our study focused on the principal VOC elevated by PLRV infection and did not consider possible effects of minor components in the blend. At least one study that used herbivore-induced synomones indicates that minor compounds could be important in affecting behavior (Gouinguené and Turlings 2005), and minor components can affect activity of pheromones (Arn et al. 1985). Nonetheless, the activity of our synthetic blends is comparable to that observed with the natural blend trapped from headspace, suggesting we have isolated the most important components required for activity in this system.

Our direct observational data also confirm that activity requires a blend. A few differences in time budgets were observed among aphids exposed to individual compounds, but distinct and significantly different behavioral patterns were observed in aphids exposed to either a synthetic blend that consisted of all individual compounds or headspace volatiles collected from PLRV-infected plants. Over PLRV blends, the aphids spent less time walking, and their mean walking speed was lower than aphids over non-infected blends. In aphids, host plant selection may be divided into four consecutive steps: (1) prealighting behavior, (2) leaf surface exploration and probing of subepidermal tissues, (3) deep probing of plant tissues, and (4) evaluation of the phloem sap (Vargas et al. 2005; Powell et al. 2006). The relative importance of each of these stages may be affected by the degree of host specialization (Bernays and Funk 1999) and by the species studied (Tosh et al. 2003; Vargas et al. 2005). Our results have implications for the second stage in the host-finding behavior of aphids, which involves leaf surface exploration and probing of subepidermal tissues. During this stage, aphids exhibit antennal waving on the plant surface, evidently facilitating detection of odors in the boundary layer close to the plant surface (Hardie and Powell 2000). Differences in emigration, time allocation to walking, and walking speed in our study indicate that the preference for PLRV-infected plants exhibited by the green peach aphid permitted to contact, probe, and feed on potato plants (Castle et al. 1998) is at least in part determined by aphid responses to volatile cues available near the plant surface.

Whether other modalities influence the response of aphids to PLRV-infected plants remains to be determined.

For all our experiments, we used a clonal population of green peach aphid based on a collection made in southern Idaho. Our results with this clone show that the species is capable of responding differently to blends than to pure compounds from host plants. The vast areas planted to potatoes in southern Idaho and the superior quality of PLRV-infected plants for green peach aphid growth and reproduction (Castle and Berger 1993) may have selected for responsiveness to the specific compounds and blends we studied. Responses of other populations of green peach aphid may differ, and the phenomenon should be examined in other green peach aphid clones.

Our results assist in understanding disease spread. Arrestment of the green peach aphid to PLRV-infected plants can increase the probability of virus acquisition promoting further the spread of PLRV, in addition to the observed payoff of increased fitness (Castle and Berger 1993). McElhany et al. (1995) infer from modeling studies that examined effects of preference for infected or healthy plants on pathogen spread, that the rate of pathogen spread depends on the percentage of infected plants, and that rates of pathogen spread would be high when vectors prefer healthy plants. However, their model leaves out other factors such as insect density, insect movement, or the initial numbers of inoculative insects, which may also play a role in rates of pathogen spread. From our study and other studies (Eigenbrode et al. 2002; Jiménez-Martínez et al. 2004; Srinivasan et al. 2006), it is beginning to emerge that volatiles emitted by infected plants contribute to this preference. In the future, it will be important to examine these and other factors that may be causing such preferences and their relative importance in disease spread. Conducting such experiments is time consuming and costly. We thus suggest that these and other factors be incorporated in models to fully understand the consequences of the observed preferences on disease spread.

Acknowledgments This research was supported by USDA-NRI Competitive Grant No.: 2003-35302-13354. We thank William Price for statistical assistance, and Brent Werner, Karla Medina-Ortega, and Melia Nafus for technical assistance. This is a publication of the Idaho Agricultural Experiment Station.

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Introduction

Coupled gas chromatography–electroantennographic detection (GC-EAD) is a widely used procedure for distinguishing insect olfactory stimulants within mixtures of volatile chemicals. Insect antennae are exposed to compounds eluting from a gas chromatograph (GC) while the electrical potential across them is continuously recorded (Arn et al. 1975). Voltage deflections are detected in the antennae coincident with the elution of olfactory stimulants, and stimulants of particular biological importance for the insect, such as pheromones, typically produce relatively larger deflections. The elicitation and comparative strength of antennal responses can be used to assess the likelihood that a particular compound will be found (after further investigation) to have behavioral activity for an insect. GC-EAD has been used effectively by researchers to rapidly screen large numbers of candidate semiochemicals or raw extracts of active material for further evaluation in behavioral experiments, thereby eliminating unnecessary behavioral tests of inactive compounds (Cork et al. 1990; Bjostad 1998).

The utility of EAD data depends greatly upon the quality of the signal detected from the antenna and the signal-to-noise ratio. Noise can be generated within the antennal preparation and apparatus by sources of electromagnetic radiation, changing local electric fields, changes in the quality of electrode contact with the antenna, amplifier noise, as well as movement of the antennae caused by air fluctuations, muscular contractions, or mechanical vibrations conducted through the equipment (Van der Pers 1998). This constant stream of random background noise and spurious electrical perturbations invariably mask some true signals (i.e., potentials presumably arising from stimulated olfactory sensilla), and otherwise compete with them for recognition (Fig. 1). Generally, authors have reported EAD-detected olfactory stimulation without indicating a specific method for discriminating signal from noise. In some cases, authors have stipulated "consistency" or "repeatability" of an EAD spike at specific retention times as evidence for an olfaction-generated signal (Ellner et al. 2001; Nunez et al. 2002). To improve the repeatability of EAD data interpretation, some have employed explicit tests based in the statistical improbability of a random noise spike both exceeding a threshold level and recurring at the same retention time (Zhang et al. 2001; Zhang and Aldrich 2003; Asaro et al. 2004). Such methods gain power through repeated runs of the same compound over the same insect species. However,

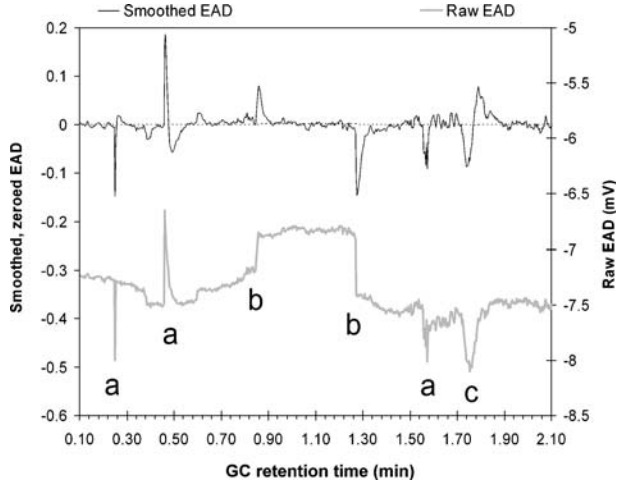


Fig. 1 A composite EAD trace illustrating various types of deflections that may be present. The lower trace is the raw unfiltered EAD trace as it is recorded from the antenna, and the upper trace has had zero-filtering and some smoothing applied. Non-signal "spikes" are shown at locations *a*, baseline shifts are shown at *b*, and an olfaction generated signal is shown at *c*. Smaller random noise perturbations are seen throughout

because these methods discriminate signals using deflection amplitude data alone without considering deflection shape, they may require many data runs to resolve genuine olfactory responses, and they may be ineffective at distinguishing signals when noise-associated deflections are similar in amplitude.

The signal-to-noise ratio in EAD procedures can sometimes be improved with modifications to the associated hardware and antennal preparation technique. For example, the signal-to-noise ratio of an EAD preparation that utilizes a single antenna can be improved by attaching multiple antennae in parallel to the recording electrodes (Park and Baker 2002). In addition, the unique dynamics of olfactory responses can be exploited in the use of filters that discriminate between signal and noise. Voltage deflections produced by antennae exposed to olfactory stimulants have a characteristic shape and duration (or wavelength) that is the product of both the dose/time characteristics of the presented stimulus and antenna physiology (Sullivan and Slone, unpublished data). Consequently, low-pass filters have been incorporated into EAD amplifiers that attenuate short wavelength signals typical of electromagnetic noise while passing the long (i.e., >1–10 sec) wavelengths associated with electrophysiological responses (Guerin et al. 1983; Bjostad 1998; Sasaerila et al. 2000; Leal et al. 2001). The analog EAD signal output is commonly converted to a digital stream for data storage and examination with graphical software. With the data recorded as a series of numbers representing the antennal voltage measured at a fixed interval (e.g., every 0.2 sec), mathematical filters can also be applied to antennogram data sets post-collection to extract antennal response from background noise. Such post hoc methods are available on commercial EAD software and include smoothing algorithms such as moving-average which, like low-pass filters, tend to attenuate noise with shorter wavelengths. However, the shape dynamics of olfaction-generated EAD signals are adequately distinct to allow discrimination based on more precise waveform characteristics; hence, further refinement to such post hoc noise filters is possible. Assuming the concentration of stimulant eluting from the GC is not approaching the saturation point of the olfactory sensillae, EAD output amplitude will covary approximately linearly with the log-transformed concentration of an eluting stimulant (Zhang et al. 2002; Sullivan, unpublished data) or the first integral of the amplitude of the flame ionization detector output. Hence, the shape of the raw olfaction-generated signal closely resembles that of the flame ionization detector (FID) peak. Under normal GC operating conditions (i.e., the column is not overloaded and its temperature is programmed to increase constantly), these peaks should resemble the bell shape of a normal distribution (Fig. 2). Hence, EAD peaks arising from GC-generated stimuli eluting will have an essentially constant wavelength

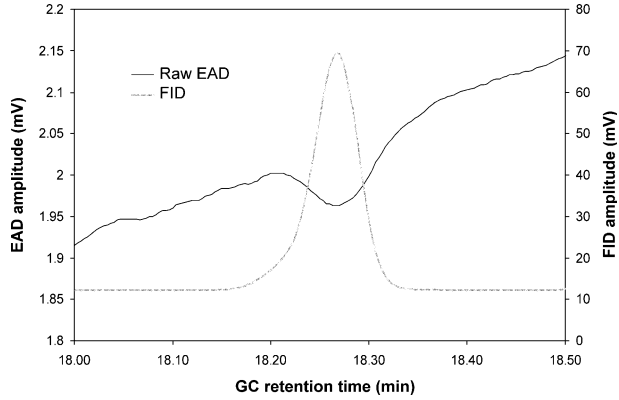


Fig. 2 A GC peak and associated EAD olfaction response superimposed to show the approximate bell shape of each

(i.e., peak width) and a high degree of bilateral symmetry between their left and right slopes.

In this paper, we describe three algorithms that discriminate olfaction-generated signals from noise in EAD data on the basis of either (1) signal amplitude alone, (2) signal amplitude and wavelength, or (3) signal amplitude, wavelength, and symmetry. We then compare the signal-discriminating capacity of the three algorithms to one another and to discrimination methods based on either general visual appearance of EAD traces or retention time consistency of signals. The algorithms are formulated to be applicable to any EAD data sets where voltage changes across antennae are detected with a high-impedance DC amplifier (such as described in Bjostad 1998 and used in EAD systems sold by Syntech, Hilversum, The Netherlands) interfaced with an analog-to-digital converter and data management software that allows the final collected data set to be converted to an ASCII file and examined in a spreadsheet program. PC-based digital data acquisition systems suitable for GC-EAD commonly permit the user to convert recorded data to ASCII format (including data acquisition systems sold by Syntech; SRI Instruments, Torrance, CA, USA; and DATAQ, Akron, OH, USA), so it should be possible for most GC-EAD operators to apply our algorithms to their own data sets. In addition, we have made available on the Internet an Excel spreadsheet that performs all of the calculations.

Methods and Materials

Apparatus Setup and Data Collection EAD data were collected from the antennae of the Nantucket pine tip moth, *Rhyacionia frustrana* (Comstock; Lepidoptera: Tortricidae), the southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae), and the parasitoid *Roptrocerus xylophagorum* (Ratzeburg; Hymenoptera: Pteromalidae) using procedures and apparatus described previously (Pettersson et al. 2000; Asaro et al. 2004; Sullivan 2005). Antennae were mounted between two glass electrodes filled with Beadle–Ephrussi ringer solution amended with polyvinylpyrrolidone (Bjostad 1998), and the recording electrode was connected to a high impedance guarded input AC/DC preamplifier (Syntech). Output from the preamplifier was processed by a signal interface box (Syntech IDAC 2/3) that provided optional baseline control of the analog signal. Digitized signal from the FID and the signal interface box were collected at five samples per second with a PeakSimple Chromatographic Interface and PeakSimple software (SRI instruments). Data were collected without the automatic baseline return filter engaged on the IDAC 2/3. The EAD and FID data files were saved in ASCII format and subsequently opened in an Excel spreadsheet as two columns of successive voltage readings from the FID and the EAD [note that for data collected with Electro Antenno Detection software (Syntech 2004), "EAD session" data files (*.ead) can be saved in ASCII format (*.asc) within the "File: Save As" window].

Data Transformation We first applied an exponential zeroing filter to the raw EAD data. Filtrations of the data in this way converted the approximately Gaussian raw EAD signals to "sigmoid" deflections that were essentially identical to the output produced by the analog baseline-return filter present on the SYNTECH IDAC 2/3 signal interface box:

$$Z_t = E_t - E_{t-1} + r \cdot Z_{t-1} \quad (1)$$

where Z=zeroed data, t=sample number, E=EAD data (i.e., voltage recorded from antenna), and r=zeroing rate [related to the time constant (T.C.) on the SYNTECH IDAC 2/3 by $r = 0.37^{\frac{1}{f_s \cdot TC}}$; f_s=sampling rate per second. We set r to a value of $0.8w^{0.05}$ where w is

the width in samples of an average FID peak in the sample run at half its height. This value produced positive and negative deflections in the zeroed EAD data that were similar in amplitude.

We further filtered the zeroed data to remove spurious noise spikes by applying an exponential averager (Lyons 1997):

$$s_t = \alpha_s \cdot Z_t + (1 - \alpha_s) \cdot s_{t-1}, \tag{2}$$

where α_s=weighting factor for smoothing. The purpose of the averaging filter is to pass deflections that occur at the wavelength expected of an olfaction signal (for EAD data, this is the wavelength of the FID peaks) while attenuating the higher frequencies associated with noise. For typical GC-EAD recordings where the FID peak width at half-height is 2–3 sec (10–15 time steps), we found that a value of 0.15 for α_s maximized the difference in amplitude between signal peaks and higher frequency noise, thus allowing for the highest S/N ratio.

We calculated a baseline using a similar exponential averaging function:

$$b_t = \alpha_b \cdot s_t + (1 - \alpha_b) \cdot b_{t-1}, \tag{3}$$

where α_b=weighting factor for the baseline. The baseline is the axis from which we measured height of EAD deflections. Hence, it ideally should attenuate signals with the wavelength expected from olfaction signals while passing lower frequencies, thereby allowing the baseline to follow the average meanderings of the zeroed EAD data and permitting the subtraction of baseline "drift". To achieve this end, we set α_b to 0.001.

Signal Discrimination We transformed the data finally with one of three candidate algorithms designed to "filter out" EAD deflections that did not possess specific distinguishing characteristics of those produced by olfactory stimulants eluting from the GC. Our goal was to design a function whose value (ψ) was large and positive when an olfaction-generated signal occurred at retention time t, and small or negative otherwise.

The first algorithm was the *Peak Height method*. The amplitude of negative EAD deflections from the baseline was measured at time t. This algorithm formalized the process ostensibly used by EAD operators during visual evaluation of graphic EAD output, namely, locating negative deflections in zeroed data that stand out appreciably from the background noise:

$$\psi_{P(t)} = b_t - s_t, \tag{4}$$

The second algorithm was the *Additive method.* In addition to the initial downward deflection of the EAD output, a second positive deflection is generated by the zeroing filter (Eq. 1) when the raw data stream contains a negative-deflecting Gaussian peak of a wavelength similar to the GC peaks. The amplitude of this positive deflection will equal approximately that of the negative deflection when the EAD response possesses the same wavelength (i.e., peak-width) as a GC-generated stimulus (i.e., an FID peak), when r of the zeroing algorithm is set to $0.8w^{0.05}$. Hence, for EAD signals generated by GC-eluting stimuli, that is, "true" olfactory responses, the sum of the amplitudes of a negative deflection occurring at time t and a corresponding positive deflection occurring at time $t+w$ will have a value that exceeds that produced by random background noise comprised of random frequencies:

$$\psi_{A(t)} = (b_t - s_t) + (s_{t+w} - b_{t+w}). \tag{5}$$

This algorithm tends to filter out EAD deflections with either low amplitude and/or inappropriate wavelength, and therefore, was hypothesized to be more accurate at

discriminating signal from noise than the "Peak Height" algorithm (Eq. 4) which discriminated signals by using amplitude alone.

Finally, in the *Minimum method*, we recorded the smallest of the negative and positive deflections of the zero-filtered EAD data stream at the wavelength expected from an olfaction-generated peak as the value of the function:

$$\psi_{M(t)} = \min\left[(b_t - s_t), (s_{t+w} - b_{t+w})\right]. \tag{6}$$

This method discriminated specifically against EAD deflections in the zeroed data where the negative deflection at time t and positive deflection at $t+w$ differed substantially from each other in amplitude: higher function values are generated for peaks with both the symmetry of a Gaussian peak *and* appropriate wavelength. Because this algorithm discriminated peaks based on three diagnostic qualities of olfaction-generated EAD signals (i.e., peak amplitude, wavelength, and symmetry), we hypothesized that this algorithm might result in greater rejection of noise than the two previously described.

Noise Characterization In the absence of compounds eluting from the GC, EAD output had a continuous and predictable noise pattern. The distribution of noise around the baseline was normally distributed. In addition, we found that the individual points had a high degree of positive serial correlation, with first degree correlations typically above 0.8 (Slone and Sullivan, unpublished data). Serial correlation in the data peaks would invalidate traditional statistical techniques by violating the assumption of independence.

Signal and Noise Measurement We defined a "peak" as any location where the following was true:

$$\psi_t = \max\left(\psi_{t-2}, \psi_{t-1}, \psi_t, \psi_{t+1}, \psi_{t+2}\right). \tag{7}$$

By using only the peaks, we reduced the amount of points to process and eliminated the ~0.8 first-order serial correlation present in EAD data (Slone and Sullivan, unpublished data). These peak values were normally distributed around their mean, which allowed us to use conventional statistical techniques.

To categorize each peak as either signal or noise, we first identified sections of the processed EAD run (ψ) where elution of olfactory stimuli from the GC could not have occurred. These sections included retention times both before the solvent peak eluted from the column (but after the initial antenna noise had abated) and after all analytes had eluted at the end of the GC temperature program. We then calculated the mean (μ_n) and standard deviation (σ) of all positive peaks within those sections as an estimate of baseline noise inherent in the entire EAD trace.

Next, a cutoff amplitude was selected to separate true signal peaks above the cutoff from noise peaks below the cutoff. This amplitude was set through a combination of the noise distribution and the desired probability of committing a type I (false identification of noise as signal) error. The experimentwise error probability (α_E; typically 0.05) depended upon the number of peaks in the data set and the probability that any single peak might trigger a type I error (comparisonwise error, α_c). The α_E and α_c values are related by the following equation, functionally similar to the Bonferroni correction (Jones 1984):

$$\alpha_c = 1 - (1 - \alpha_E)^{1/n}, \tag{8}$$

where n is the total number of peaks in the portion of the data stream that is being tested. The cutoff amplitude was calculated by finding the value of the z statistic that satisfied the

following equation of the normal distribution populated with the calculated values of α_c and σ. (e.g., Mendenhall et al. 1990):

$$\alpha_c = \int_{y=z}^{\infty} \frac{1}{\sigma\sqrt{2\pi}} \exp\left[-\left(\frac{1}{2\sigma^2}\right)y^2\right], \tag{9}$$

and adding that value to the mean noise level (μ_n). Any peak that had a value greater than (μ_n+z) was categorized as a signal, and all others were rejected as noise.

Performance Testing: Simulation We evaluated the sensitivity and noise-rejection capacity of the algorithms by applying them to simulated EAD data sets that contained a specified amplitude of random background noise and either a simulated olfaction signal (a normally distributed deflection of known amplitude and a width at half-height of w), a spurious, short-duration deflection event (spike), also of known amplitude, or an instantaneous baseline-shift of known amplitude (see Fig. 1). For each simulation, t_{max}=3,000, with 1,000 samples containing only background noise (used for measuring μ and σ of the noise) and 2,000 samples containing background noise plus the simulated signal/spurious event, and each simulation was replicated 20 times The specific tests we performed were as follows:

1. Twenty well-spaced simulated olfaction signals with background noise repeated at several signal/noise ratios
2. Positive and negative simulated spurious spikes randomly generated at a probability of 0.0125 per time step (~25 spikes per run; these spikes were randomly generated to allow for any effects from varied spacing and positive–negative spike interactions). These runs were also repeated with the spikes occurring over a range of amplitudes.
3. Instantaneous positive and negative baseline shifts, randomly generated at a probability of 0.0125 (~25 shifts per run; the shifts were randomly generated for reasons described in 2 above).

Performance Testing: Real Data We applied the algorithms to real data sets and compared their performance. First, we examined EAD runs from *R. frustrana*, *D. frontalis*, and *R. xylophagorum* and then compared FID and EAD traces at retention times where the different algorithms indicated antennal signals.

Second, we applied the algorithms to noisy EAD runs of *D. frontalis* females exposed to analyzed extracts of hindguts of *D. frontalis* males. Eleven electrophysiologically active molecules had been previously identified for this combination of insect and test extract (Sullivan 2005). We processed 17 individual EAD runs using experimental error rates (α_E) from a conservative 2.7e-6 to a liberal 0.9999. For each run, we calculated the baseline noise level and cutoff amplitude using EAD output after the solvent peak, but before the first FID-detectable sample peak eluted from the GC, and again after no further peaks were detected by the FID. We noted the number of signals detected by each algorithm that matched the retention times of the 11 known active molecules and also any signals detected at other retention times. Similarly, we performed 17 replicates with each algorithm where the digital EAD output from two runs selected at random from the 17 *D. frontalis*/hindgut runs were averaged, then 17 replicates of four runs averaged, 17 replicates of eight runs averaged, and finally one replicate of all 17 runs averaged. These runs were all processed with experimental error rates (α_E) of 0.05. This method of averaging multiple EAD runs

from different antennae to improve the signal-to-noise ratio was previously used by Sullivan (2005).

Results and Discussion

Simulation Testing Tests of the three algorithms that used simulated EAD runs revealed the strengths and weaknesses of each, with highly significant performance differences ($P<<0.05$) found among the algorithms in all tests. The Peak Height method was always inferior in finding signals to the other two methods, and the Additive method was significantly better than the Minimum method (Fig. 3). In terms of noise rejection, the Peak Height method was also inferior to the other two methods in cases where the spike amplitude was less than eight times, or the baseline shift amplitude was less than three times, the maximum background noise amplitude (99th percentile of all noise peaks). With very large spikes or baseline shifts, the Additive method rejected less noise than the other methods. The superior method for noise rejection was clearly the Minimum method, which rejected large spikes and baseline shifts five to ten times better than the other methods.

The simulation testing was deliberately designed to highlight marginal cases where olfaction-generated signals were competing with large amounts of background noise. For EAD runs where the amplitude of olfaction-produced deflections stands well beyond the background (i.e., those performed with especially good antennal preparations and more-than-adequate concentrations of stimulants in the test sample), most signals would be easily discerned by any of the algorithms or by eye. However, there are often cases where the amplitudes of some or all of the signals are small, or the number of spurious deflection events in the run are large, and in these situations, the Additive or Minimum methods should provide EAD operators with a powerful tool for improving their ability to discriminate noise from signal. If the operator's goal is to identify all possible signals in a run with the possibility of encountering some false positives, then the Additive method is preferable. If the goal is to minimize errors, then the Minimum method is superior. The discriminatory power of the algorithms can be enhanced several-fold if applied to data from

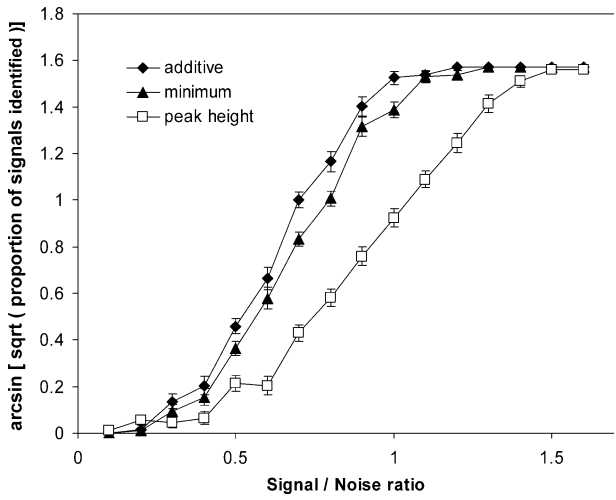


Fig. 3 The proportion of simulated signals that each of the algorithms discriminated is shown as the mean of 20 replications plus standard deviation error bars at each of several signal to noise ratios. An arcsin (sqrt) transformation was used to linearize the proportional results. For S/N ratios <1.5, the Additive method was slightly superior to the Minimum method, and both were far better at discriminating the simulated signals than the Peak Height method. For S/N ratios ≥1.5, all methods discriminated all signals

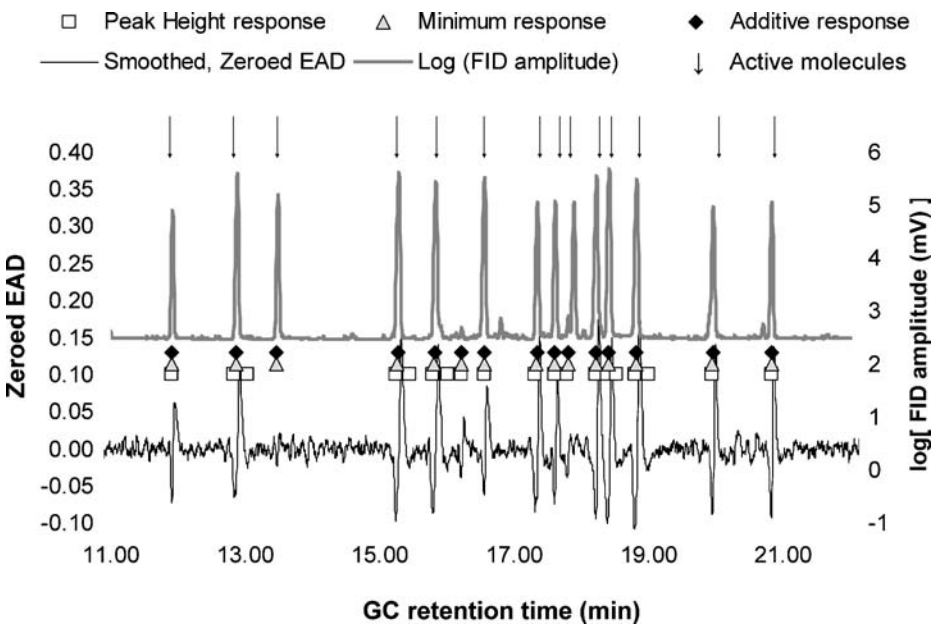


Fig. 4 Zeroed EAD recording of the parasitic wasp *Roptrocerus xylophagorum* (Hymenoptera: Pteromalidae) and the associated GC trace from a mix of host and associated plant odors. The locations of previously determined active molecules (*arrow*) and significant responses (Additive, *diamond*; Minimum, *triangle*; Peak Height, *square*; α_E=0.05) from the three algorithms are shown

multiple GC-EAD runs (replications) because the likelihood that a "false" signal will be registered at the same retention time will become vanishingly small with additional replication. Statistical tests can also be performed on the frequency that signals are detected at a particular retention time to further reject spurious signals and accept only the retention times of active compounds; signals should occur with a greater frequency than predicted by random chance at the retention times of active molecules (Zhang et al. 2001; Zhang and Aldrich 2003; Asaro et al. 2004). The chief contribution of these two algorithms to interpretation of EAD runs is the increase of the effective signal/noise ratio in the data, an improvement which reduces the number of EAD runs necessary for a researcher to accurately discern electrophysiologically active compounds.

Real Data Testing All three algorithms successfully discriminated antennal signals from noise in EAD data from the three orders of insects examined. In an EAD recording of the parasitic wasp *R. xylophagorum* responding to a mix of host and associated plant odors, the Additive and Minimum methods correctly "tagged" all of the active compounds [i.e., compounds whose activity with olfactory sensillae had been demonstrated by statistical analysis of multiple EAD runs (Pettersson et al. 2000; Sullivan, unpublished) in the mixture (Fig. 4)]. The Peak Height method failed to detect one active compound and returned five apparent false positives at the tail end of active compounds. All methods identified a compound at 16.21 min that has not previously been demonstrated as an active molecule. In a rather noisy EAD recording of the southern pine beetle, *D. frontalis*, subjected to the same mix of odors as *R. xylophagorum* above (Sullivan, unpublished), all three methods identified signals at all three retention times of compounds with demonstrated olfactory and/or behavioral activity with this beetle (Fig. 5). Additionally, all three methods identified

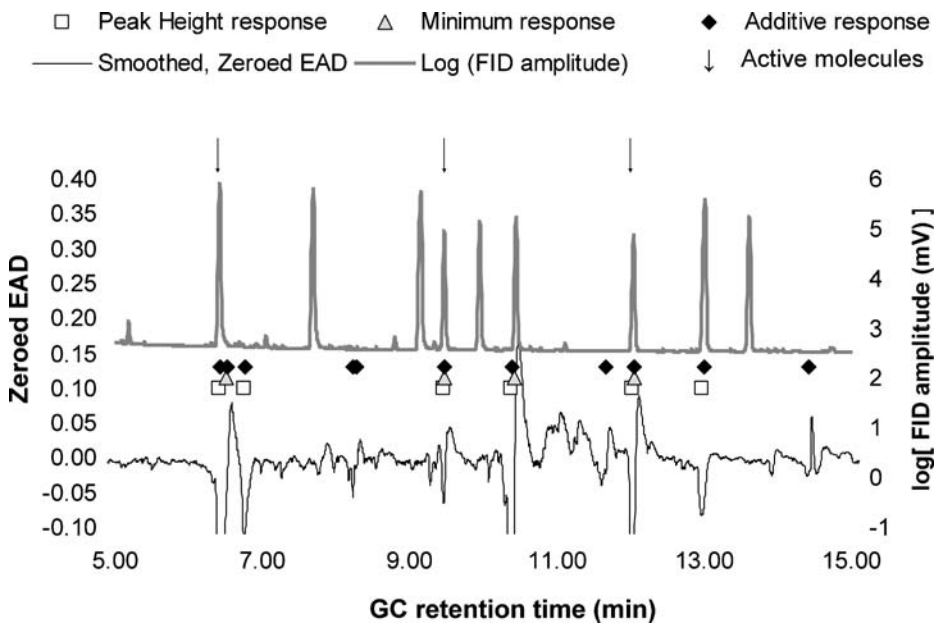


Fig. 5 Zeroed EAD recording of the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Curculionidae) subjected to the same mix of odors as *R. xylophagorum* in Fig. 6. The locations of previously determined active molecules (*arrow*) and significant responses (Additive, *diamond*; Minimum, *triangle*; Peak Height, *square*; α_E=0.05) from the three algorithms are shown

a rather large peak at approximately 10.50 min associated with the elution of *p*-cymene, although this has not previously been demonstrated to be an olfactory stimulant. While the Minimum method returned no additional positives, the Additive method detected seven additional active retention times that were apparently false positives, and the Peak Height method returned two additional false positives. In an EAD recording of a female Nantucket pine tip moth (*R. frustrana*) exposed to a mixture of host-associated odors (Asaro et al. 2004), all three algorithms correctly identified signals at retention times of seven known olfactory stimulants in the mixture (Fig. 6). The Additive method also detected one additional active molecule at 15.70 min that has not been identified previously as an olfactory stimulant for this insect, and it returned an additional two false positives. The Peak Height and Minimum methods failed to detect two known active molecules, and the Peak Height method returned a single false positive. All algorithms also identified one FID peak at approximately 16.50 min that had not been shown previously to be an active compound.

The replicated EAD runs from *D. frontalis* females that responded to male *D. frontalis* hindgut extract were relatively noisy. In general, approximately half of the retention times associated with electrophysiologically active compounds were identified at α_E=0.05, but with clear differences in performance among the algorithms (Fig. 7). The Additive method consistently identified more "true" signals than the other two algorithms at any α_E level, yet tagged fewer erroneous retention times than the Peak Height method. The Minimum method identified fewer signals than the other methods, but showed greater error-rejecting capability by not tagging any retention times incorrectly at α_E levels$\leq$0.05.

Although the Minimum method had superior error-rejection capacity, the Additive method showed the best overall power by consistently producing the fewest false

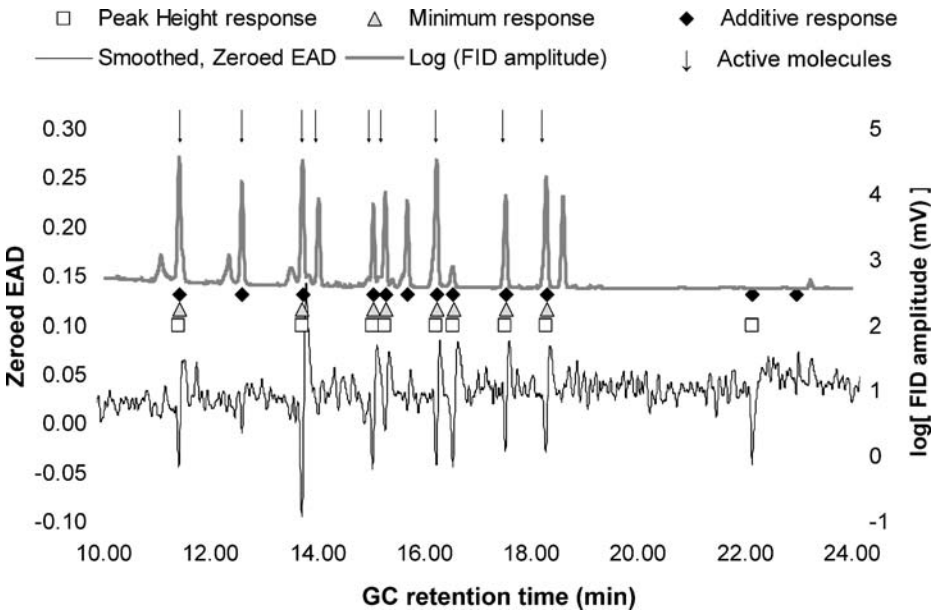


Fig. 6 Zeroed EAD recording of a female Nantucket pine tip moth, *Rhyacionia frustrana* (Lepidoptera: Tortricidae) and the associated GC trace from a mixture of host-associated odors. The locations of previously determined active molecules (*arrow*) and significant responses (Additive, *diamond*; Minimum, *triangle*; Peak Height, *square*; α_E=0.05) from the three algorithms are shown

classifications per correct classification (Fig. 8). The Minimum method had medium overall power, and the Peak Height method had the lowest power with real data sets, returning the highest number of false positives per true response. Algorithm traces for the Additive method also consistently had higher average signal/noise ratios than output from the other algorithms (Fig. 9). Additionally, the Additive method recognized more true olfaction-generated signals than is apparently possible by visually comparing peak heights in EAD

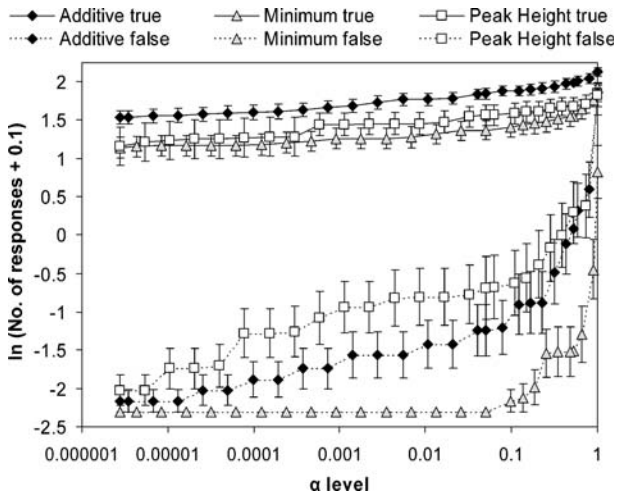


Fig. 7 Average number of true and false significant responses (±1 SD) from 17 southern pine beetle EAD runs produced by each algorithm using a range of experimental error rates (α_E). The numbers of signals found were log(x+0.1)-transformed before averaging to normalize the variances

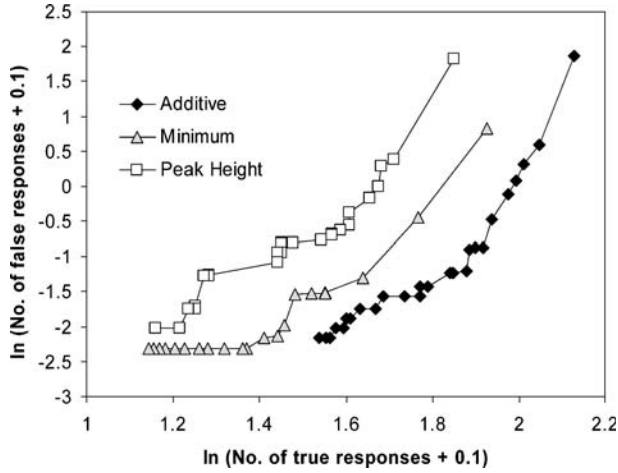


Fig. 8 True vs. false significant responses returned from each algorithm for southern pine beetle EAD runs. Each point shown represents the average of 17 runs evaluated at a different α_E level, ranging from 2.7e-6 (*farthest left*) to 0.9999 (*farthest right*). Points lower and further to the *right* indicate superior performance

traces, as this latter procedure was ostensibly duplicated by the Peak Height algorithm. Thus, our data demonstrate that the Additive algorithm was an effective "amplifier" of olfaction-generated responses in insect-produced EAD traces.

Our analyses of averaged data from randomly selected combinations of EAD replicates showed that greater signal-resolving power and improved noise rejection occurred when the algorithms were applied to averaged data from progressively greater numbers of runs

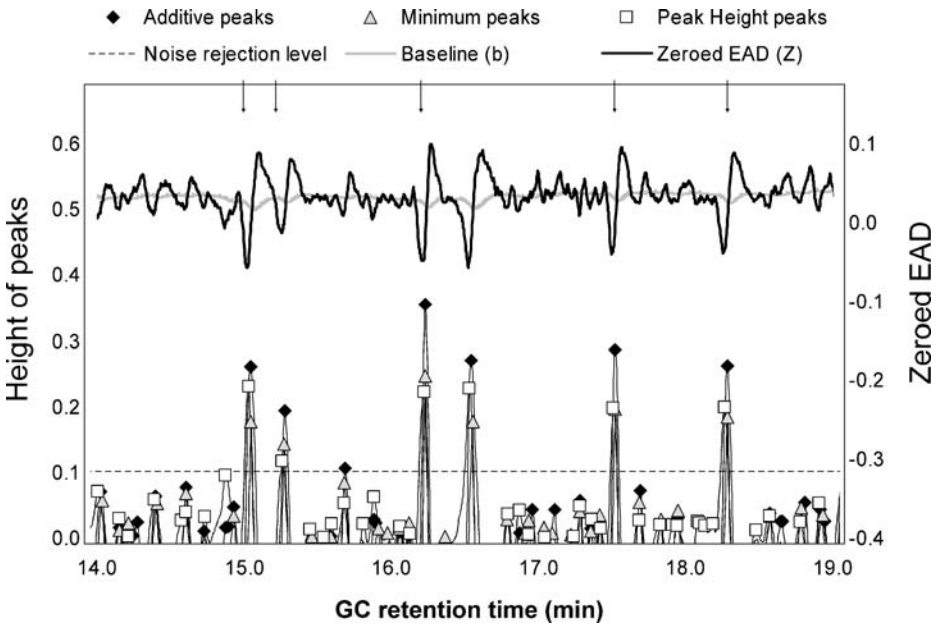


Fig. 9 Output traces (ψ) from each of the algorithms are shown below a section of EAD output from Fig. 6, a female Nantucket pine tip moth, *Rhyacionia frustrana* (Lepidoptera: Tortricidae) responding to a mix of host-associated odors. The output from each method has been standardized so that the mean of the trace is 0, and the noise rejection limit is 0.1. The Additive method amplified EAD olfaction peaks relative to noise peaks more than the Minimum or Peak Height methods

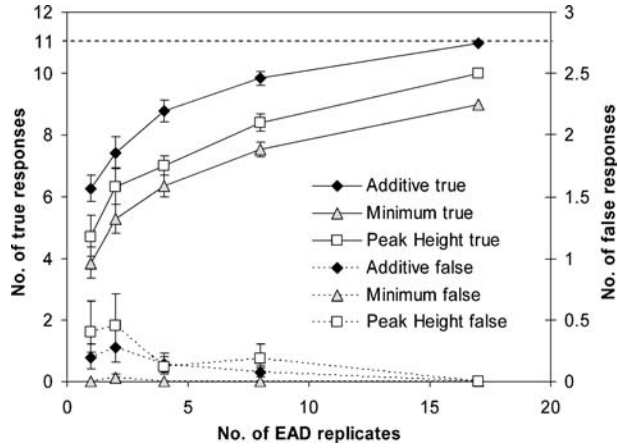


Fig. 10 Results from southern pine beetle EAD run averaging showing increasing numbers of true responses and decreasing numbers of false responses when more runs were averaged. With all 17 runs averaged, the Additive method found all 11 active molecules with no errors. Data were log(x+0.1)-transformed for analysis and back-transformed for display. Note that the true and false responses are displayed on different scales for clarity

(Fig. 10). With α_E=0.05, the Additive method consistently identified the greatest number of retention times associated with active compounds while committing a modest number of errors, and it was the only method to tag the retention times of all 11 active molecules when all 17 runs were averaged. When four or more runs were averaged, the Additive method also consistently identified a location at 22.75 min corresponding to an unknown singly oxygenated monoterpene. Because this was an unknown compound with uncertain biological activity, it was excluded from the analysis and was not counted as either correct or incorrect. The Peak Height method identified a moderate number of active retention times, but it also had the highest number of apparent false positives. The Minimum method identified the fewest signals, but it generally had no false positives. Because the EAD traces of southern pine beetle responding to hindgut extracts were generally noisy, several replicated analyses of averaged run sets were necessary to resolve all 11 active molecules. Nonetheless, the analysis in Fig. 10 demonstrates that both the Minimum algorithm and, to a greater extent, the Additive algorithm can reduce the number of EAD runs necessary for discerning electrophysiologically active compounds.

Applications Our Additive algorithm represents a powerful tool that can be used to enhance the realized signal-to-noise ratio of any GC-EAD system that stores its data digitally. Raw data can be copied into a spreadsheet program, transformed, and then examined using the graphics capabilities of the spreadsheet. We created an Excel 2000 spreadsheet that contains all of the required transformations and calculations. The user pastes raw or pre-zeroed EAD data into a column, selects areas of the data with no eluting molecules to calibrate the noise level, and enters other information about the data collection methodology. Charts on the spreadsheet display the traces and indicate significant impulses found by each of the three methods. This spreadsheet is available as electronic supplementary material (doi:10.1007/s10886-007-9338-6).

In summary, with a novel insect or compound, the optimal use of GC-EAD data for identifying olfactory stimulants is of great importance, as an incorrect identification of a stimulant could lead a researcher seeking behavior-modifying compounds to waste effort on an inactive compound; in contrast, failure to recognize evidence of olfactory stimulation could cause an important behavioral molecule to be overlooked. For antennal preparations of some insect species, the maximum signal level obtainable may be at or below the

background noise level (for example, see Russell et al. 2004). As our Additive and Minimum method algorithms have the capacity for extracting signals that are similar in amplitude to surrounding noise, they should effectively increase the sensitivity of an EAD system and may allow data collection where it would not otherwise have been possible. Additionally, our algorithms and data analysis procedures eliminate the potential for experimenter bias that is inherent in visual evaluation of data sets because the decision of whether a deflection event is a "real" signal or not is made by a uniformly applied mathematical test. By combining these algorithms with replication, high levels of power and discrimination for detecting active molecules in EAD data are provided.

Acknowledgments The bulk of this work was completed at the USDA Forest Service Forest Insect Research facility while the first author was employed there. The authors thank two anonymous reviewers for their thoughtful comments.

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geometric isomers of the 2,13-dienal revealed specific attraction to a lure with the (2*E*,13*Z*)-isomer as a main component.

Keywords Sex pheromone · Octadecadienal · Reversed-phased HPLC · APCI-LC-MS analysis · 2,4-Dinitrophenylhydrazone · Lepidoptera · Sesiidae · *Macroscelesia japona*

Introduction

Moths in the family Sesiidae have clear wings and a wasp-mimetic body. Although most of the clearwing moths are diurnal and possibly communicate with visual cues, the females produce a species-specific sex pheromone that attracts males. It is well known that sesiid pheromones are mainly composed of 2,13- and 3,13-octadecadien-1-ols and their acetates (Ando et al. 2004; Ando 2007; El-Sayed 2007). In addition, recent studies have reported 2,13-octadecadienal as a component of sesiid pheromones. For example, Francke et al. (2004) identified the (2*E*,13*Z*)-isomer from *Sesia apiformis*, and we identified the same compound from *Macroscelesia japona* and *M. longipes* (Naka et al. 2007). These structure determinations, however, were reported without any information as to other geometric isomers of the 2,13-dienal. GC-MS analyses of the pheromone extracts were carried out by using only one synthetic compound with the 2*E*,13*Z* configuration as an authentic standard, and chromatographic behaviors of the other geometric isomers have not been reported. Activities of the isomers have never been evaluated in the laboratory or in the field. Studies with all geometric isomers of the 2,13-dienal are necessary to confirm the identification. Furthermore, the Sesiidae includes more than 1,000 species worldwide (Pühringer and Kallies 2004). The diversity of the insects suggests that another possible pheromone component, 3,13-octadecadienal, also might be found in the Sesiidae. Therefore, we synthesized all geometric isomers of the 2,13- and 3,13-dienals and characterized them. Because most isomers of the dienals were unstable to GC conditions, HPLC and LC-MS analyses were used instead. We also reexamined a pheromone extract of *M. japona* to confirm our previous identification. The results of this paper should be useful for unraveling other pheromone systems in the Sesiidae.

Materials and Methods

Instruments ^{1}H and ^{13}C NMR spectra were recorded with a Jeol Alpha 500 spectrometer at 500.2 and 125.7 MHz, respectively, with $CDCl_3$ solutions containing TMS as an internal standard. GC analyses were carried out on an HP-5890 Series II gas chromatograph (Hewlett-Packard, Wilmington, DE, USA) equipped with a flame ionization detector (FID) and a DB-23 column (0.25 mm ID×30 m, 0.25 μm film, J & W Scientific, Folsom, CA, USA). The column temperature program was 50°C for 2 min, 10°C/min to 160°C, then 4°C/min to 260°C. Samples were injected via a cool on-column injector heated at oven temperature plus 3°C after holding at 53°C. The carrier gas was He. GC-MS was conducted in EI mode (70 eV) with an HP5973 mass spectrometer system (Hewlett-Packard) equipped with the same DB-23 column as the GC analysis and a split/splitless injector. The carrier gas was He, and the ion source temperature was 230°C. HPLC analyses were made with a Jasco PU-980 liquid chromatograph equipped with an integrator (System Instrument Chromatocorder 21J), a UV detector (Jasco UV-970, 235 or 210 nm), and an ODS column (Senshu Pak PEGASIL ODS-II, 2.0 mm ID×25 cm; Senshu-kagaku, Tokyo, Japan). As the

eluent, 16 % H_2O in acetonitrile was used at a flow rate of 0.2 ml/min. LC-MS was conducted with a Shimadzu LCMS-2010 equipped with an atmospheric pressure chemical ionization (APCI) interface and the same ODS column and eluent as for the HPLC analysis. The mass spectra were recorded in negative ion mode, and the following parameters were used: APCI temperature (400°C), CDL temperature (250°C), and nebulizer gas flow rate (2.5 l/min).

Chemicals The chemical structures of the 2,13- and 3,13-dienyl compounds are abbreviated as follows: Z=(*Z*)-double bond, E=(*E*)-double bond, number before the hyphen=position of the double bond, number after the hyphen=carbon number of the straight chain, Ald=aldehyde, and OH=alcohol. All geometric isomers of octadecadienals were prepared from the corresponding alcohols, which had been synthesized in our previous study (Naka et al. 2006), by the following oxidation.

Oxidation of Octadecadien-1-ols with (E)-2-Double Bond E2,Z13-18:OH (300 mg, 1.1 mmol) in CH_2Cl_2 (10 ml) was mixed with pyridinium chlorochromate (PCC, 300 mg) and stirred 4 h at room temperature. After addition of hexane (30 ml), precipitates were removed by filtration, and the filtrate was concentrated. The residue was taken up in hexane (30 ml), and insoluble precipitates were filtered off. After evaporation of hexane in vacuo, the residue was chromatographed on silica gel to give E2,Z13-18:Ald (190 mg, 63 %). By the same procedure, E2,E13-18:Ald was obtained from E2,E13-18:OH.

Dess-Martin Oxidation of Other Octadecadien-1-ols Z2,Z13-18:OH (100 mg, 0.38 mmol) in CH_2Cl_2 (1 ml) was added to a solution of Dess-Martin periodinane (Dess and Martin 1991; 500 mg, 1.2 mmol) in CH_2Cl_2 (4 ml) at room temperature. After stirring for 3 h, the mixture was diluted with ether (20 ml), and the resulting mixture was poured into saturated aqueous $NaHCO_3$. The organic layer was separated, washed with water, dried over Na_2SO_4, concentrated in vacuo, and purified by column chromatography with silica gel to give Z2,Z13-18:Ald (75 mg, 75%). By the same procedure, Z2,E13-18:Ald and the four geometric isomers of 3,13-octadecadienal were obtained from the corresponding alcohols. NMR analyses of Z2,Z13-18:Ald and Z2,E13-18:Ald (see Tables 1 and 2) showed trace contamination (~3%) from the corresponding aldehyde with the (*E*)-2-double bond, indicating slight isomerization of the (*Z*)-2-double bond. In the case of the 3,13-octadecadienal, isomerization of the (*Z*)-3- and (*E*)-3-double bonds was barely detectable by NMR analysis.

Derivatization of Octadecadienals with 2,4-Dinitrophenylhydrazine Each isomer (10 μg) of the dienals was separately coated in a glass vial (2 ml) and treated with 2,4-dinitrophenylhydrazine (1 mg) in H_2O (200 μl) at room temperature. After 1 h, the resulting hydrazone derivative was extracted with hexane (400 μl×3). After removal of the hexane, the residue was dissolved in acetonitrile and analyzed by LC-MS. The derivatization was also carried out for a mixture of four geometric isomers (50 ng each) of the 2,13-dienal or 3,13-dienal with a small amount of hydrazine in a similar manner.

Insects and Pheromone Extraction Ten larvae of *Macroscelesia japona* were collected in November in Maibara City, Shiga Prefecture, and kept in a refrigerator at 8±1°C without light for 2 months. They were then placed in a plastic cage at 25±1°C under a 15L:9D photoperiod until emergence as adults (Naka et al. 2007). Abdominal tips of the females that adopted a calling position were excised and extracted with hexane for 15 min.

Field Tests The synthetic lures were tested in two field trials: Takatsuki City (Osaka, Japan) from June 14 to July 8, 2006, and Tsukuba City (Ibaraki Prefecture, Japan) from June 15 to July 1, 2006. A rubber septum (white rubber, 8 mm OD, Sigma-Aldrich, St. Louis, MO, USA) was used as a dispenser of the synthetic lure and placed in the center of a sticky board trap (30×27 cm bottom plate with a cover, Takeda Chemical Ind., Osaka), which was set 1.5 m above the ground.

Results

Synthesis and Structure Confirmation of Dienals E2,Z13-18:Ald and E2,E13-18:Ald were easily synthesized by PCC oxidation of the corresponding alcohols, E2,Z13-18:OH and E2, E13-18:OH. Because the same oxidation of other geometric isomers of 2,13-octadecadien-1-ol and all isomers of 3,13-octadecadien-1-ol did not yield the required aldehydes at all, we examined Dess-Martin oxidation with a periodinate [1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one] (Dess and Martin 1983, 1991). This reagent had been used in the successful oxidation of alkenols with a double bond in the 2- or 3-position (Schwarz et al. 1986), and we confirmed its efficacy for the oxidation of the 2,13- and 3,13-dienols.

Table 1 shows the ^{1}H NMR data of all geometric isomers of 2,13- and 3,13-dienals, which were synthesized without isomerization of the double bond. The 2,13-dienal could be readily distinguished from the 3,13-dienal by the splitting pattern of the aldehyde proton (1-H). The signal of the 2,13-dienal was a doublet, and that of the 3,13-dienal was a triplet. Additionally,

Table 1 ^{1}H NMR assignments for the characteristic signals of 2,13- and 3,13-octadecadienals

Position	Chemical Shift (ppm), [coupling constant (Hz)]			
	(*Z,Z*)-isomer	(*E,Z*)-isomer	(*Z,E*)-isomer	(*E,E*)-isomer
2,13-octadecadienal (2,13-18:Ald)				
1 (1H, d)	10.08 [8.3][a]	9.50 [7.9][a]	10.08 [8.3][a]	9.50 [7.9][a]
2 (1H, dd)	5.96 [11.0][b]	6.12 [15.6][b]	5.96 [11.2][b]	6.12 [15.5][b]
3 (1H, dt)	6.64 [8.3][c]	6.85 [6.8][c]	6.63 [8.3][c]	6.85 [6.9][c]
4 (2H, dt)	2.61	2.33	2.60	2.34
5 (2H, m)	1.50	1.50	1.50	1.51
12, 15 (4H, m)	~2.02	~2.02	~1.97	~1.98
13, 14 (2H, m)	5.35	5.35	5.38	5.39
18 (3H, t)	0.90	0.90	0.89	0.88
3,13-octadecadienal (3,13-18:Ald)[d]				
1 (1H, t)	9.65 [1.8][a]	9.65 [2.1][a]	9.66 [1.8][a]	9.65 [2.2][a]
2 (2H, dd)	3.18 [7.3][b]	3.11 [6.6][b]	3.18 [7.3][b]	3.11 [6.6][b]
3 (1H, dt)	5.55 [10.8][c]	5.49 [15.4][c]	5.53 [10.8][c]	5.49 [15.3][c]
4 (1H, dt)	5.71	5.62	5.71	5.62
5 (2H, m)	~2.04	~2.06	2.03	2.06

[a] $J_{1\text{-}2}$

[b] $J_{2\text{-}3}$

[c] $J_{3\text{-}4}$

[d] NMR data of the olefinic and allylic protons from 12- to 15-positions and terminal methyl protons at the 18-position were almost the same as those of the corresponding 2,13-18:Ald

the 2,13-dienal showed two allylic methylene protons (4-H_2) at δ 2.3-2.6 ppm, whereas the 3,13-dienal showed two allylic methylene protons (2-H_2) at δ 3.1-3.2 ppm, at lower field because of the proximity to the carbonyl group. Because chemical shifts of the olefinic protons at the 2- and 3-positions of the 2,13-dienal and at 3- and 4-positions of the 3,13-dienal were different from those at the 13- and 14-positions, the splitting patterns were readily interpretable to indicate configuration of the double bonds at the 2- and 3-positions. Coupling constants between the two olefinic protons of the (*Z*)- and (*E*)-double bonds were ~15.5 and ~11 Hz, respectively. ^{13}C NMR data were also informative in confirming the structures of the synthetic dienals, because many signals were recorded without overlapping (Table 2). The 2,13-dienal was distinguished from the 3,13-dienal by its characteristic allylic carbon (2-C) at δ 43-47 ppm. Because the resonance of the carbonyl carbon (1-C) was affected by conjugation, the positional isomers could be differentiated by comparing the chemical shifts of 1-C. In addition, the chemical shift of each olefinic and allylic carbon at the (*Z*)-double bonds was upfield from that of the corresponding carbon at the (*E*)-double bonds without exception, clearly indicating the geometric configuration of each isomer.

GC Analysis of the Synthetic Dienals The four geometric isomers of each dienal, whose structures and purities had been confirmed by NMR, were analyzed by GC on a DB-23

Table 2 ^{13}C NMR assignments for the characteristic signals of 2,13- and 3,13-octadecadienals

Position	Chemical Shift (ppm)			
	(*Z,Z*)-isomer	(*E,Z*)-isomer	(*Z,E*)-isomer	(*E,E*)-isomer
2,13-octadecadienal (2,13-18:Ald)				
1	191.0	194.2	191.0	194.2
2	130.2	133.0	130.2	133.0
3	153.5	159.1	153.5	159.1
4	28.0	32.8	28.0	32.8
5		27.9		27.9
5–11 or 6–11[a]	29.1–29.7	29.2–29.8	29.1–29.6	29.1–29.6
12[b]	26.9	26.9	32.3	32.3
13[c]	129.8	129.8	130.30	130.29
14[c]	129.9	129.9	130.36	130.35
15[b]	27.2	27.2	32.6	32.6
16	32.0	32.0	31.8	31.8
17	22.4	22.4	22.2	22.2
18	14.0	14.0	14.0	14.0
3,13-octadecadienal (3,13-18:Ald)[d]				
1	199.7	200.3	199.7	200.4
2	42.6	47.3	42.6	47.3
3	117.9	119.0	117.9	119.0
4	135.5	137.0	135.5	137.0
5	27.6	32.7	27.6	32.7

[a] Signals from C-5 to C-11 for (3*Z*,13*Z*)- and (3*Z*,13*E*)-isomers, and from C-6 to C-11 for (3*E*,13*Z*)- and (3*E*,13*E*)-isomers

[b,c] Chemical shift values might be reversed

[d] Chemical shifts of the carbons from the 6- to 18-positions were almost the same as those of the corresponding 2,13-18:Ald

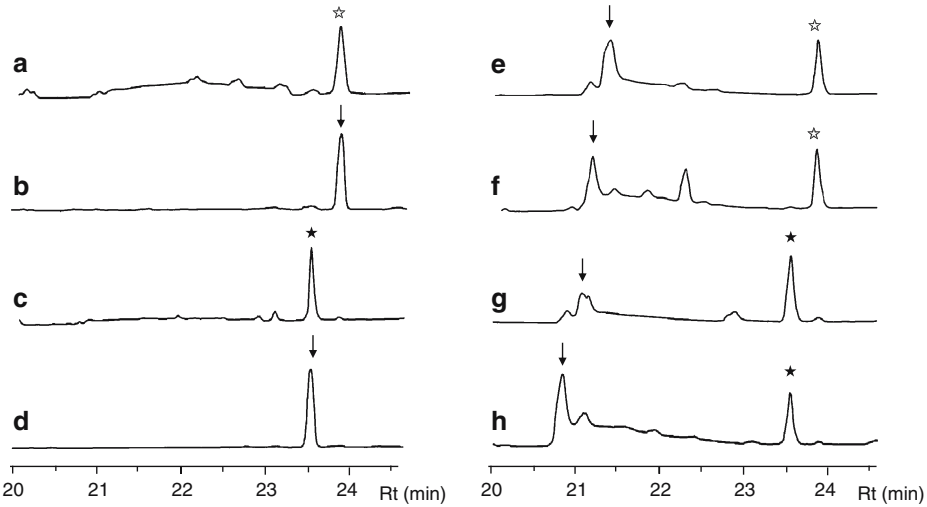


Fig. 1 GC analysis of synthetic 2,13- and 3,13-octadecadienals (20 ng each) on a DB-23 column (0.25 mm ID×30 m) with cool on-column injection; **a** Z2,Z13-18:Ald, **b** E2,Z13-18:Ald, **c** Z2,E13-18:Ald, **d** E2,E13-18:Ald, **e** Z3,Z13-18:Ald, **f** E3,Z13-18:Ald, **g** Z3,E13-18:Ald, and **h** E3,E13-18:Ald. The *arrows* show the compounds that were expected to be detected without any isomerization of the double bonds, and the identifiable isomerized products are indicated by *open stars* (E2,Z13-18:Ald) and *filled stars* (E2,E13-18:Ald)

capillary column with cool on-column injection. The FID chromatograms are shown in Fig. 1, and tentatively assigned retention times (R_ts) are listed in Table 3. On a polar column, an aldehyde usually elutes faster than the corresponding alcohol and acetate. However, R_t of E2,Z13-18:Ald was longer than R_ts of E2,Z13:OH and E2,Z13:OAc (see footnote in Table 3) because of the conjugation of the aldehyde. In this analysis, the observed R_ts of Z2,Z13-18:Ald and E2,Z13-18:Ald were the same (Fig. 1a and b), and those of Z2,E13-18:Ald and E2,E13-18:Ald were also the same (Fig. 1c and d) indicating complete conversion of the (*Z*)-2-double bond into the (*E*)-2-double bond under the GC conditions used. In the case of the 3,13-dienal, each geometric isomer showed a complex chromatogram (Fig. 1e–h). Z3,Z13-18:Ald and E3,Z13-18:Ald produced a recognizable peak at the R_t of E2,Z13-18:Ald (23.86 min), and Z3,E13-18:Ald and E3,E13-18:Ald produced a peak at the R_t of E2,E13-18:Ald (23.53 min), indicating the partial isomerization of the (*Z*)-3- and (*E*)-3-double bonds into the (*E*)-2-double bond. In addition to the peak with the long R_t, each geometric isomer of the 3,13-dienal showed some broad peaks with shorter R_ts (21-22 min) indicating complicated isomerization of the double bond at the 3-position. With reference to the R_ts of the corresponding alcohols and acetates (see footnote in Table 3), we assumed that the peaks indicated by arrows were four geometric isomers of the 3,13-dienal eluted from the GC column that had kept their original structures. If this is the case, the isomers of 3,13-dienal eluted in the order *EE* → *ZE* → *EZ* → *ZZ*. In the course of GC analysis with a split/splitless injector, the (*Z*)-3- and (*E*)-3-double bonds were completely isomerized to the (*E*)-2-double bond.

HPLC Analysis of the Dienals and LC-MS Analysis of Their Hydrazones The synthetic dienals were first analyzed by HPLC with an ODS column. The 2,13-dienal with a conjugated system was perceptible at a ng-level with a UV detector operated at 235 nm (Fig. 2a). Whereas E2,Z13-18:Ald and Z2,E13-18:Ald were not completely separated, a mixture of the four isomers showed four distinguishable peaks. The elution order was *ZZ* → *EZ* → *ZE* → *EE*.

Table 3 Chromatographic behaviors of synthetic 2,13- and 3,13-octadecadienals on GC and HPLC and of their 2,4-dinitrophenylhydrazone derivatives on LC-MS

		R_t (min)		
		Aldehyde		Hydrazone
		GC[a]	HPLC[b]	LC-MS[c]
2,13-Diene	(*Z,Z*)-isomer	23.89[d]	33.2	25.1
	(*E,Z*)-isomer	23.86	36.0	27.6
	(*Z,E*)-isomer	23.53[d]	36.8	26.9
	(*E,E*)-isomer	23.53	39.9	29.5
3,13-Diene	(*Z,Z*)-isomer	21.42[e]	31.6	21.4
	(*E,Z*)-isomer	21.15[e]	33.7	22.8
	(*Z,E*)-isomer	21.08[e]	34.5	22.8
	(*E,E*)-isomer	20.86[e]	36.7	24.4

[a] Retention times (R_ts) on a DB-23 column (0.25 mm ID×30 m, 0.25 μm) using a cool on-column injector. Column temperature: 50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 230°C. R_ts (min) of some corresponding alcohols and acetates were as follows: E2,Z13-18:OH 22.29 , E2,Z13-18:OAc 21.82, E3,Z13-18:OH 21.61, E3,Z13-18:OAc 21.37

[b] R_ts on a ODS column (2.0 mm ID×25 cm). Eluent: 16 % water in acetonitrile. Flow rate: 0.2 ml/min. Detection: UV 235 nm for 2,13-dienal and 210 nm for 3,13-dienal. λ_{max} of four geometric isomers of 2,13-dienal were as follows: Z2,Z13-18:Ald 229 nm, E2,Z13-18:Ald 222 nm, Z2,E13-18:Ald 227 nm, and E2,E13-18:Ald 223 nm

[c] R_ts on an ODS column (2.0 mm ID×25 cm). Eluent: 8% water in acetonitrile. Flow rate: 0.2 ml/min. APCI detected a negative ion at *m/z* 443 ($[M-1]^-$ of hydrazones derived from the aldehydes) in selected ion monitoring mode

[d] Completely isomerized to E2,Z13-18:Ald or E2,E13-18:Ald during GC analysis (see Fig. 1a–d)

[e] Peaks expected to be not isomerized. Samples were mainly isomerized to E2,Z13-18:Ald, E2,E13-18:Ald, and other unknown compounds under the GC conditions used (see Fig. 1e–h)

Although the 3,13-dienals were detected by UV at 210 nm (Fig. 2b), the sensitivity was low and the peaks were broad. The elution order of the four geometric isomers of the 3,13-dienal was the same as that of the 2,13-dienal. Each isomer of the 3,13-dienal eluted faster than the corresponding isomer of 2,13-dienal possessing the same configuration (Table 3). Next, these dienals were analyzed by LC/MS after derivatization with 2,4-dinitrophenylhydrazine using the same ODS column. Each derivative was sensitively detected by negative ion APCI. Fig. 2d and e shows the mass chromatograms monitoring $[M-1]^-$ at *m/z* 443 of the 2,13- and 3,13-dienals, respectively. The hydrazone of Z2,E13-18:Ald eluted faster than that of E2,Z13-18:Ald, and thus, the elution order of the 2,13-dienal derivatives was $ZZ \rightarrow ZE \rightarrow EZ \rightarrow EE$. Hydrazones of the 3,13-dienal exhibited a similar elution order, and the derivatives of E3,Z13-18:Ald and Z3,E13-18:Ald eluted at almost the same R_ts (Table 3). The chromatogram in Fig. 2d was recorded for a 1:1:1:1 mixture of four geometric isomers. However, the peak areas of the hydrazones of Z2,Z13-18:Ald and Z2,E13-18:Ald were smaller than the others owing to partial conversion of the (*Z*)-2-double bond into the (*E*)-2-double bond during derivatization. This derivatization was achieved with the hydrazine dissolved in distilled water at room temperature. The reaction with an acidic aqueous solution of the hydrazine, which was used as a general procedure for various aldehydes, caused easy conversion of the configuration. Further mild conditions for the hydrazone formation were examined, but cooling and dilution of the hydrazine solution did not affect the final ratio of the products. We could not find a set of conditions that prevented the isomerization completely. Isomerization

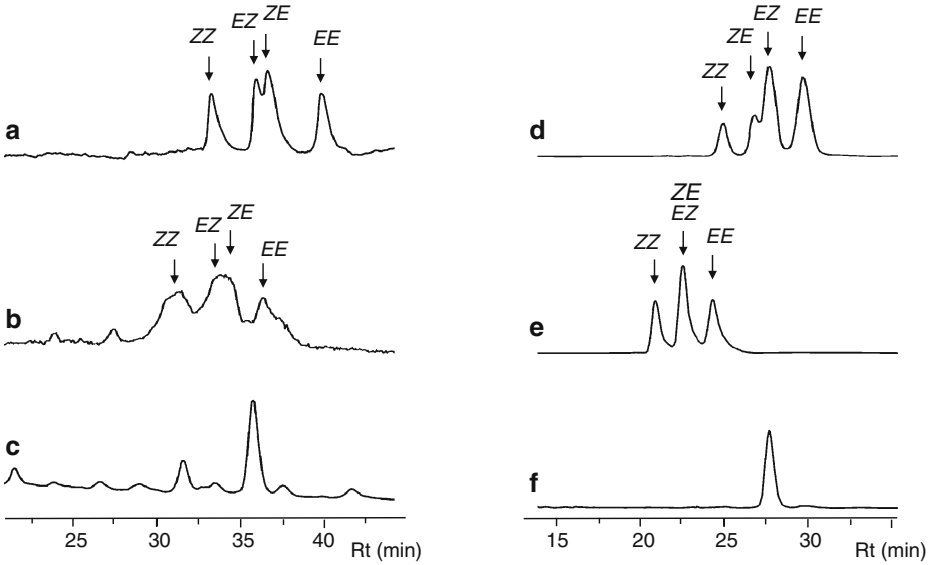


Fig. 2 HPLC analysis of octadecadienals (**a**–**c**, UV absorption) and LC-MS analysis of their 2,4-dinitrophenylhydrazone derivatives (**d**–**f**, mass chromatograms of $[M-1]^-$ at *m/z* 443); **a** a mixture of four geometric isomers of 2,13-octadecadienal (50 ng each, UV 235 nm), **b** a mixture of four geometric isomers of 3,13-octadecadienal (250 ng each, UV 210 nm), **c** a crude pheromone extract of *M. japona* (5 FE), **d** hydrazones derived from the four isomers of 2,13-octadecadienal (50 ng each), **e** hydrazones derived from the four isomers of 3,13-octadecadienal (50 ng each), and **f** a crude pheromone extract of *M. japona* (2.5 FE) treated with the hydrazine. Both analyses used the same column (ODS, 2.0 mm ID×25 cm), eluent (16% H_2O in acetonitrile), and flow rate (0.2 ml/min)

of the double bond at the 3-position to the (*E*)-2-double bond was also observed but was slight, as shown in Fig. 2e.

Reexamination of the Dienal Produced by M. japona Females Previous GC-MS analysis had indicated that a crude extract of abdominal tips from *M. japona* females included an octadecadienal and an octadecadien-1-ol in a ratio of 10:1 (Naka et al. 2007). To confirm the structure of the aldehyde component, a pheromone extract of five female equivalents (FE) was analyzed by HPLC with the ODS column that had been used for the analysis of authentic dienals, operated under the same conditions. As shown in Fig. 2c, the extract included a component with the same R_t as authentic E2,Z13-18:Ald (36.0 min) among other unknown compounds detected at UV 235 nm. Several fractions were recovered, and an aliquot of each fraction was analyzed by GC-MS. This analysis showed E2,Z13-18:Ald only in a fraction that included the component eluted at 36.0 min. The rest of the fraction was analyzed by LC-MS after treatment with 2,4-dinitrophenylhydrazine, and the hydrazone derivative of E2,Z13-18:Ald was detected. Furthermore, another extract (five FE) was directly treated with the hydrazine and then examined by LC-MS. As shown in Fig. 2f, the hydrazone of a dienal was detected at the same R_t as the derivative of E2,Z13-18:Ald (27.6 min) but not at the R_t of the 3,13-dienal. These experiments confirmed that E2,Z13-18:Ald detected by the previous GC-MS analysis was not an artifact from degradation of Z2,Z13-18:Ald or the 3,13-dienal.

Field Attraction of M. japona Males Whereas attraction of *M. japona* males with a lure baited with a 10:1 mixture of E2,Z13-18:Ald and E2,Z13-18:OH was found in our previous

Table 4 Attraction of *Macroscelesia japona* males to traps baited with a binary synthetic lure including 2,13-octadecadienal (2,13-18:Ald, major component) and 2,13-octadecadien-1-ol (2,13-18:OH, minor component)[a]

Lure (mg/septum)		Captured males/trap[b]	
2,13-18:Ald (0.5)	2,13-18:OH (0.05)	Takatsuki[c]	Tsukuba[d]
(*E,Z*)-Isomer	(*E,Z*)-Isomer	10.7±5.0 a	3.0±1.4 a
(*E,Z*)-Isomer	(*Z,Z*)-Isomer	6.3±2.9 a	6.3±4.8 a
(*E,Z*)-Isomer	(*Z,E*)-Isomer	1.0±0.8 b	1.0±0.0 b
(*E,Z*)-Isomer	(*E,E*)-Isomer	0	0
(*Z,Z*)-Isomer	(*E,Z*)-Isomer	0	0
(*Z,E*)-Isomer	(*E,Z*)-Isomer	0	0
(*E,E*)-Isomer	(*E,Z*)-Isomer	0	0
None		0	0

[a] Compounds with the *E,Z* configuration (E2,Z13-18:Ald and E2,Z13-18:OH) are the insect-produced isomers

[b] Mean±SE. Values within each test followed by a different letter are significantly different at $p<0.05$ by Tukey-Kramer test

[c] Captured at three copses in Takatsuki City in Osaka Prefecture, Japan, from June 14 to July 8, 2006. In each copse, one trap was used

[d] Captured at three copses in Tsukuba City in Ibaraki Prefecture, Japan from June 15 to July 1, 2006. In each copse, one trap was used

field tests (Naka et al. 2007), the activity of their geometric isomers had not been evaluated. Therefore, several mixtures combining the insect-produced isomers with other isomers were tested. Thus, E2,Z13-18:Ald (natural major component) was mixed with a geometric isomer of E2,Z13-18:OH as a minor component, and E2,Z13-18:OH (natural minor component) was mixed with a geometric isomer of E2,Z13-18:Ald as a major component. The results are shown in Table 4. The population density of the insects was not high in 2006, but the natural-type lure baited with E2,Z13-18:Ald and E2,Z13-18:OH attracted male moths. In addition to this combination, lures baited with E2,Z13-18:Ald and Z2,Z13-18:OH also attracted some moths. Lures baited with other combinations of aldehyde and alcohol were not attractive.

Discussion

Based on the experiments that used all four geometric isomers of 2,13- and 3,13-octadecadienals as authentic standards, we propose a procedure for the identification of an aldehyde component in pheromone extracts of sesiid moths. Because many lepidopteran sex pheromones are composed of multiple components with different functional groups, and because GC-MS generally detects alcohols and acetates with high sensitivity and without isomerization, a crude pheromone extract should first be analyzed by GC-MS. However, even by using a cool on-column injector, dienal samples with a (*Z*)-2-, (*Z*)-3-, or (*E*)-3-double bond will be partly to completely converted into the isomer with an (*E*)-2-double bond. Isomerization will be complete with a split/splitless injector, giving one peak for the isomerized aldehyde, which might be better than the cluster of peaks obtained with the cool on-column injector for the preliminary examination of the pheromone extract. When an octadecadienal is detected, the procedures described here can confirm the position and

geometry of the double bonds. In our previous experiments with the *M. japona* pheromone, we reduced the aldehyde component with $NaBH_4$ and analyzed the resulting alcohol by GC-MS to determine the double bond position and configuration in the parent aldehyde (Naka et al. 2007). As an alternative approach, the structure determination can be accomplished by HPLC and LC-MS with an ODS column. When the pheromone component is a 2,13-dienal, the configuration of the two double bonds can be directly determined by the HPLC chromatographic behavior monitored at UV 235 nm. In the case of a 3,13-dienal, where a trace amount of the natural pheromone cannot be detected by UV monitoring, APCI-LC-MS analysis of the hydrazone derivative can be used to determine the configuration of the double bonds. Although R_ts of the hydrazones derived from Z3,E13-18:Ald and E3,Z13-18:Ald were almost the same, the configuration at the 13-position of the original compound is determined by the above preliminary GC-MS analysis. During GC analysis, Z3,E13-18:Ald and E3,Z13-18:Ald are expected to be converted into stable E2,E13-18:Ald and E2,Z13-18:Ald, respectively, which are separable on a polar GC column.

In this study, we confirmed the E2,Z13 configuration of the aldehyde secreted by *M. japona* females. In addition to the sesiid species, E2,Z13-18:Ald has been identified from a tineid species, *Tineola bisselliella* (Yamaoka et al. 1985). However, the unstable Z2,Z13-18:Ald has never been reported from pheromone gland extracts, whereas the corresponding alcohol component (Z2,Z13-18:OH) has been identified from some sesiid species, along with E2,Z13-18:OH (Ando 2007; El-Sayed 2007). The homo-conjugated double bond of a 3,13-octadecadienal is also unstable and easily migrates to the 2-position. The 3,13-dienal might be produced by enzymatic oxidation of the 3,13-dien-1-ol, which has been identified from several species as a pheromone component. Interestingly, (*E*)-2-Hexenal and (*Z*)-3-hexen-1-ol are well known as a leaf aldehyde and a leaf alcohol, respectively. Many plants also produce unstable (*Z*)-3-hexenal, another leaf aldehyde, which acts as an attractant to some predatory insects (Shiojiri et al. 2006). It would be interesting to know whether moth species utilize the relatively unstable 3,13-dienal as a sex pheromone. Szöcs et al. (1989) reported that males of two tineid species, *Monopsis rusticella* and *Nemapogon falstriellus*, were attracted by lures that included Z3,Z13-18:Ald. This finding suggests that some lepidopteran species might actually secrete the 3,13-dienal. Conversely, it is possible that the unstable aldehyde attracted the males after isomerization of the (*Z*)-3-double bond. We are now carrying out screening tests with the synthetic dienals to find new attractants for sesiid species in Japan.

Acknowledgement We are grateful to Mr. R. Wakasugi and Mr. D. Wakatsuki in Zoological Laboratory of Meijo University for the collection of *M. japona* and Dr. F. Mochizuki of Shin-Etsu Chemical Co., for supplying some dienyl compounds. This work was supported in part by a research grant from Heart Co. (Tokyo, Japan).

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pheromone antagonist in *L. dispar* and why (−)-disparlure reduces pheromonal attraction of male *L. dispar* but not male *L. obfuscata*.

Keywords Indian gypsy moth · *Lymantria obfuscata* · *Lymantria dispar* · (+)-Disparlure · (−)-Disparlure · (*Z*)-2-Methyloctadec-7-ene · (3*R*,7*Z*)-3-Methylnonadec-7-ene · (3*S*,7*Z*)-3-Methylnonadec-7-ene · (3*R*,7*R*,8*S*)-*cis*-7,8-Epoxy-3-methylnonadecane · (3*S*,7*R*,8*S*)-*cis*-7,8-Epoxy-3-methylnonadecane · Sex pheromone · Lepidoptera · Lymantriidae

Introduction

The apple hairy moth or Indian gypsy moth, *Lymantria obfuscata* Walker 1865 (Lepidoptera: Lymantriidae), is a periodic pest on various fruit trees (Beeson 1941; Rahman and Kalra 1944; Butani 1979) and forest trees (Mathur 1964; Verma et al. 1979; Chacko et al. 1988) in India. It is found in northern India (Jammu and Kashmir, Himachal Pradesh, Uttar Pradesh, Punjab), northern Pakistan (Chaudhry et al. 1966), and Afghanistan (Schintlmeister 2004), but it is not as widely distributed as previously suggested (Gupta 1992). Reports that it occurs in southern India (Kumar 1974; Misra and Choudhuri 1974) are erroneous and likely based on taxonomic confusion with *Lymantria ampla* Walker 1855 (Ramaseshiah and Ramesh Bali 1987; Chacko et al. 1988; Chacko and Singh 1990). Biology and behavior of *L. obfuscata* are described elsewhere (Beeson 1941; Masoodi 1991).

Lymantria obfuscata and allopatric *L. dispar* cross-mate under laboratory conditions and produce viable hybrids for up to three generations [Victor Mastro, United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service, Otis, MA, USA; personal comm. to PWS]. As might be expected, the sex pheromone of *L. dispar* (disparlure=*cis*-7,8-epoxy-2-methyloctadecane; Bierl et al. 1970) and analogues were shown to attract male *L. obfuscata* (Beroza et al. 1973; Punjabi et al. 1974) and were tested for population suppression (Masoodi et al. 1990).

We tested the hypothesis that the sex pheromones of *L. obfuscata* and *L. dispar* are similar. We report results of laboratory mate acceptance studies with con- and heterospecific pairings of *L. dispar* and *L. obfuscata* and the identification and field testing of sex pheromone components of *L. obfuscata*.

Methods and Materials

Experimental Insects Egg masses of *L. obfuscata* were collected in the Kulu Valley (Kashmir, India) and shipped to the Beneficial Insects Introduction Research Laboratory (BIRL), USDA, Agricultural Research Service, Newark, DE, USA, under USDA importation permit no. 36668. Larvae were reared on *L. dispar* diet (Bell et al. 1981) at 25°C, 60–75% R.H., and a photoperiod of 15L/9D. Late stage larvae of *L. dispar* were obtained from the BIRL culture, which originated from insects collected in Pennsylvania in 1976. These larvae were fed and kept as those of *L. obfuscata*.

Mate Acceptance Studies with Con- and Heterospecific L. dispar and L. obfuscata In replicated laboratory experiments 1–4, a 1- to 3-day-old male *L. obfuscata* or male *L. dispar* (BIRL colony) was paired with a virgin 1- to 3-day-old con- or heterospecific female in 500-cc paper cups (Solo Cup Co., Highland Park, IL, USA) with clear plastic lid

(Table 1). Replicates of all experiments were conducted in the afternoon hours when female moths of both species were calling. For each replicate, we recorded for up to 10 min whether and when a male attempted copulation. Differences between proportions of males attempting copulation were tested by contingency table analysis (χ^2; α=0.05) followed by Tukey-type multiple comparison of arcsin-transformed proportions (Zar 1984).

Laboratory Analyses of Pheromone Gland Extracts Abdominal tips with pheromone gland of calling 1- to 2-day-old virgin female *L. obfuscata* were removed and extracted for 5–20 min in high-performance liquid chromatography (HPLC)-grade hexane. Aliquots of pheromone gland extract and synthetic standards were analyzed by coupled gas chromatographic–electroantennographic detection (GC–EAD) and coupled GC–mass spectrometry (MS), employing equipment and general procedures previously described (Arn et al. 1975; Gries et al. 2002a). In this study, we used a Rose-5 column (30 m×0.25 mm ID×25 μm film; Rose Scientific Ltd., Edmonton, Alberta T6E 5Y5, Canada), splitless injection, a temperature program of 100°C (held 1 min) with a temperature increase of 10°C per min to 280°C, and an injector and detector temperature of 240°C.

General Methods and Instrumentation Tetrahydrofuran (THF), *N*-methylpyrrolidinone (NMP), and hexamethylphosphoramide (HMPA) were dried by standard methods. Oven-dried glassware was assembled hot and maintained under Ar; liquids were transferred by cannula under Ar pressure. Commercial anhydrous LiCl was dried by heating 2 hr at 180°C at 0.01 mm Hg and then stored in a dry box. Commercial anhydrous $MnCl_2$, CuI, and $CuCl_2$ were weighed and stored in oven-dried glassware in a dry box. Infrared spectra were recorded on Perkin-Elmer Paragon 1000 and 1600 FT-IR instruments. Optical rotations were measured on Jasco DIP-1000 and Perkin-Elmer 341 polarimeters. Nuclear magnetic resonance (NMR) spectroscopy of synthetic compounds was conducted on Bruker 300 (at 300 MHz for ^{1}H and 75 MHz for ^{13}C) and Varian AS500 (at 499.77 MHz for ^{1}H and 125.68 MHz for ^{13}C) spectrometers, with chemical shifts reported in ppm relative to TMS (^{1}H, δ 0.00) and $CDCl_3$ (^{13}C, δ 77.00). Elemental analyses were performed with a Carlo-Erba model 1106 elemental analyzer. In one instance, reaction product and unreacted starting material were separated by HPLC employing a Waters LC 625 chromatograph equipped with a Waters 486 variable-wavelength UV-visible detector set at 210 nm, HP Chemstation software (Rev.A.07.01), and a reverse-phase Nova Pak® C18 (3.9×300 mm) column (Waters) eluted with acetonitrile (1 ml/min).

Table 1 Percent of virgin 1- to 3-day-old male *L. obfuscata* or male *L. dispar* attempting to copulate, and mean time elapsed (±SD) before copulatory attempts were made, when paired with a virgin 1- to 3-day-old con- or heterospecific female in laboratory experiments 1–4 (see "Methods and Materials" for details)

Exp. No. (Replicates)	Female	Male	% Males Attempting Copulation[a]	Mean (±SD), Time (min) Elapsed Before Copulatory Attempt
1 (14)	*L. obfuscata*	*L. obfuscata*	93 a	1.38 (0.65)
2 (26)	*L. obfuscata*	*L. dispar*	15 b	1.50 (0.95)
3 (24)	*L. dispar*	*L. dispar*	83 a	1.85 (1.69)
4 (14)	*L. dispar*	*L. obfuscata*	71 a	1.67 (1.32)

[a] Numbers followed by the same letter are not significantly different; contingency table analysis of proportions of responding males (P<0.05) and Tukey-type multiple comparison of arcsin-transformed proportions (Zar 1984).

Source of Test Chemicals (*Z*)-2-Methyloctadec-7-ene (2Me-7*Z*-18Hy) and (+)- and (−)-disparlure were available from a previous project (Gries et al. 2002b). (3*R*,7*Z*)- and (3*S*,7*Z*)-3-Methylnonadec-7-enes (3*R*-Me-7*Z*-19Hy and 3*S*-Me-7*Z*-19Hy) were obtained by de-epoxidation of the corresponding (3*R*,7*R*,8*S*)- and (3*S*,7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecanes (3*R*-me-7*R*8*S*-epo-19Hy and 3*S*-me-7*R*8*S*-epo-19Hy) with triphenylphosphineselenium in dichloromethane (Clive 1978; Gries et al. 2005a).

Syntheses of (3*R*,7*R*,8*S*)- and (3*S*,7*R*,8*S*)-*cis*-7,8-Epoxy-3-methylnonadecanes

*(2'S,3'S)-cis-2',3'-Epoxy-1'-tetradecyl (1S)-10-camphorsulfonate (***2**, Fig. 1) A solution of $MnCl_2$ (3.47 g, 27.5 mmol) and LiCl (2.34 g, 55.0 mmol) in THF (55 ml) was cannulated (Ar pressure) into a 250-ml three-neck flask fitted with a low-temperature thermometer, magnetic stir bar, and pressure-equalizing 50-ml dropping funnel, and cooled to −10°C. A portion (56 ml, 80%) of a THF solution of *n*-decylmagnesium bromide [prepared from 6.09 g (27.5 mmol) of 1-bromodecane and excess Mg] was cannulated in at −10°C, followed by NMP (18 ml, 187 mmol). The mixture was warmed to 0°C, and a solution of $CuCl_2$ (38 mg, 0.28 mmol) and LiCl (24 mg, 0.56 mmol) in THF (4 ml) was cannulated in. (2′*S*,3′*R*)-4′-Bromo-*cis*-2′,3′-epoxy-1-butyl (1*S*)-10-camphorsulfonate (**1**; Aldrich; 3.00 g, 7.87 mmol) was melted *in vacuo* and dissolved in THF (10 ml). The mixture was cooled to −25° C, and the solution of **1** was added dropwise (pot temperature, −25 to −20°C). After 20 min, NMR assay of an aliquot showed complete consumption of **1**. The mixture was poured into Et_2O (100 ml) and saturated aq. NH_4Cl (100 ml). The separated organic layer was washed with H_2O (5×100 ml) and brine (25 ml), dried over Na_2SO_4, filtered, and concentrated. Column chromatography (silica gel, elution with 0–50% Et_2O-hexanes) yielded 3.30 g of impure **2** containing ring-opened alcohol. Most of the diol was removed by Kugelrohr distillation (75°C, <0.1 mm), leaving 2.77 g (79%) of nearly pure material. Purified **2**: mp 24.5–25.5°;

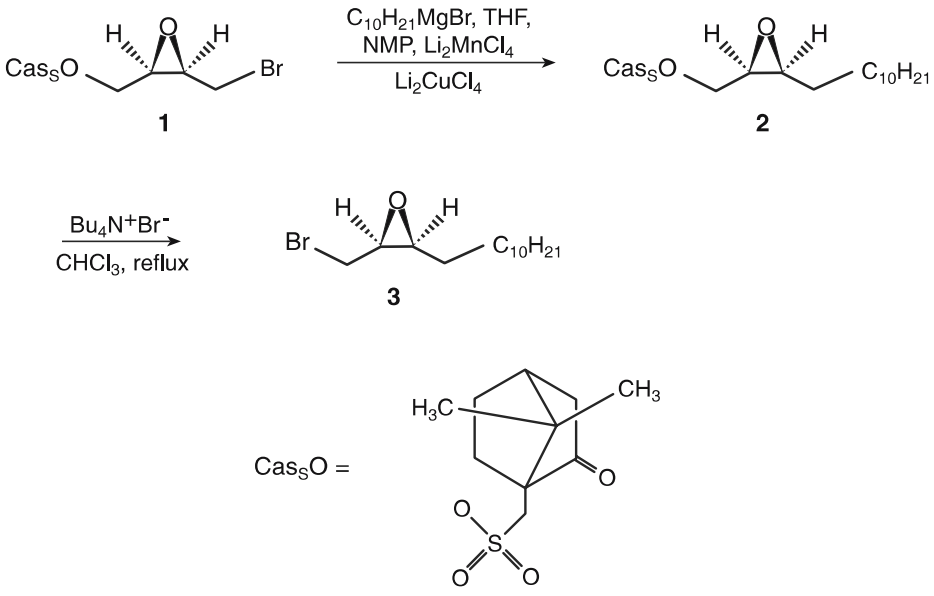


Fig. 1 Scheme for the enantioselective synthesis of (2*S*,3*S*)-1-bromo-2,3-*cis*-epoxytetradecane

$[\alpha]_D^{25} = +37.0°$ (c 2.35, $CHCl_3$); IR (film) 2920, 1747, 1360, 1172, 970 cm^{-1}; ^1H NMR δ 4.49–4.44 (dd, *J*=11.4, 4.2 Hz, 1H), 4.33–4.27 (dd, *J*=11.4, 7.1 Hz, 1H), 3.66 (d, *J*=15.1 Hz, 1H), 3.27 (m, 1H), 3.11 (d, *J*=15.1 Hz, 1H), 3.06 (m, 1H), 2.51–2.35 (m, 2H), 2.14 (t, *J*= 4.5 Hz, 1H), 2.11–2.00 (m, 1H), 1.96 (d, *J*=18.5 Hz, 1H), 1.27 (m, 18H), 1.76–1.30 (m, 4H), 1.12 (s, 3H), 0.89 (s, 3H), 0.88 (t, *J*=7.0 Hz, 3H); ^{13}C NMR 214.2, 68.5, 57.9, 56.7, 53.4, 48.0, 47.2, 42.7, 42.4, 31.8, 29.5–29.3(6), 27.9, 26.8, 26.5, 24.9, 22.6, 19.7, 19.6, 14.0. Anal. calcd. for $C_{24}H_{42}O_5S$ (%): C, 65.12; H, 9.56; found C, 65.27; H 9.85.

*(2S,3S)-1-Bromo-2,3-cis-epoxytetradecane (**3**)* Tetrabutylammonium bromide (13.8 g, 42.6 mmol) and $NaHCO_3$ (200 mg) were mixed with $CHCl_3$ [80 ml, freshly filtered through Al_2O_3; 30 ml of $CHCl_3$ was distilled off (azeotropic removal of traces of water)]. The mixture was heated to 65°C, and a solution of **2** (10.39 g, 23.5 mmol) in $CHCl_3$ (10 ml) was added. The magnetically stirred mixture was kept at 67°C for 10 hr (no **2** detected by NMR analysis), then poured into H_2O (150 ml) and pentane (200 ml). The organic layer was washed (H_2O) and dried (Na_2SO_4–$MgSO_4$). Solvent evaporation afforded a pale yellow oil, which crystallized. Column chromatography (silica gel, elution with 0–1% Et_2O-hexanes) gave 6.6 g (97%) of ~98% pure **3**. Recrystallization (pentane) gave >99% pure material: mp 35.1–35.6°C; $[\alpha]_D^{24.5} = -39.7°$ (*c*=1.9, $CHCl_3$); ^1H NMR δ 3.52–3.50 (m, 1H, H-1), 3.28–3.25 (m, 2H, H-1, H-2), 3.08–3.07 (m, 1H, H-3), 1.55–1.49 (m, 2H), 1.38–1.20 (br m, 18 H), 0.88 (t, *J*=6.6 Hz, 3H); ^{13}C NMR 58.9, 55.6, 31.8, 29.6–28.9(7), 27.2, 26.4, 22.6, 14.0. Anal. calcd. for $C_{14}H_{27}BrO$ (%): C, 57.71; H, 9.34; found C, 57.75; H 9.50.

*(R)-2′-Methylbutyl (1S)-10-camphorsulfonate (**5**, Fig. 2)* In a 500-ml 3-neck flask fitted with a dropping funnel and thermometer, a mixture of dry $MnCl_2$ (20.5 g, 163 mmol) and LiCl (13.8 g, 326 mmol) was dissolved (magnetic stirring under Ar) in THF (200 ml, 1.5 h at 0°C) to give a yellow solution (Cahiez and Marquais 1993). At −5°, 44 ml of 3M MeMgCl (132 mmol) in THF were added dropwise, followed by 108 ml (1.04 mol) of NMP at 0–5°C and then 20 ml of a THF solution of $CuCl_2$ (220 mg, 1.63 mmol) and LiCl (138 mg, 3.26 mmol) at 0° C, to give a brown-yellow solution. A solution of (*S*)-3′-bromo-2′-methylpropyl (1*S*)-10-camphorsulfonate (**4**) (>99% *ee*, Hahn et al., unpublished data; 20.0 g, 54.4 mmol) in THF (60 ml) was added dropwise at 0°C, and the mixture was held at 0°C for 16 hr; NMR analysis showed complete consumption of starting material. The mixture was poured into saturated aq. NH_4Cl and hexanes and the separated organic layer was washed with H_2O (2×100 ml) and brine. Drying over Na_2SO_4 and solvent evaporation left 16.55 g of pale yellow oil. This was dissolved in 1:1 ether/hexane, filtered through silica gel (10 g) and concentrated to leave 15.4 g (94%) of **5** as a colorless oil. Silica gel chromatography (elution with 20% ether/hexane) afforded an analytical sample: $[\alpha]_D^{26} = +36.8°$ (c 5.3, $CHCl_3$); IR (film) 2,965, 1,747, 1,358, 1,170, 963 cm^{-1}; ^1H NMR δ 4.20–4.15 (dd, *J*=9.3, 5.9 Hz, 1H), 4.09–4.03 (dd, *J*=9.3, 6.6 Hz, 1H), 3.60 (d, *J*=15.0 Hz, 1H), 2.99 (d, *J*=15.0 Hz, 1H), 2.57–2.35 (m, 2H), 2.13 (t, *J*=4.5 Hz, 1H), 2.10–2.00 (m, 1H), 1.96 (d, *J*=18.3 Hz, 1H), 1.80 (oct, *J*=6.6 Hz, 1H), 1.70–1.60 (m, *J*=4.8 Hz, 1H), 1.56–1.40 (overlapping m's, 2H), 1.34–1.16 (m, 1H), 1.12 (s, 3H), 0.98 (d, *J*=6.6 Hz, 3H), 0.93 (t, *J*=7.5 Hz, 3H), 0.89 (s, 3H); ^{13}C NMR 214.4, 74.6, 57.8, 47.8, 46.4, 42.6, 42.4, 34.5, 26.8, 26.5, 24.8, 19.7, 19.6, 15.9, 11.0. Anal. calcd. for $C_{15}H_{26}O_4S$ (%): C, 59.57; H, 8.67; found C, 59.79; H 8.72.

*(R)-1-Bromo-2-methylbutane (**6**)* Camphorsulfonate **5** (9.29 g, 30.7 mmol) and vacuum-dried tetrabutylammonium bromide (11.9 g, 36.9 mmol) were heated together to 90–95° C in a Kugelrohr distillation apparatus, starting at 1 atm pressure. The pressure was gradually reduced (needle valve) until bromide **6** began to distill; 3.51 g of 98% pure material was

collected in 0.5 hr. The semisolid pot residue was taken up in H_2O and pentane, and the pentane layer was dried over Na_2SO_4, filtered, and concentrated. Removal of solvent by distillation left another 0.89 g of **6** (total 4.40 g, 95%). Chromatography of a portion (SiO_2 column, elution with pentane) and distillation (bp 119–120°C) afforded an analytical sample: $[\alpha]_D^{20} = -6.50°$ (c 3.4, $CHCl_3$) [lit.: $[\alpha]_D^{20} = -6.66°$ (c 12, $CHCl_3$), Bestmann et al. 1998]; ^{1}H NMR δ 3.38 (dd, *J*=9.8, 5.1 Hz, 1H), 3.32 (dd, *J*=9.8, 6.1 Hz, 1H), 1.77–1.63 (m, 1H, H-2), 1.54–1.41 (m, 1H, H-3), 1.33–1.19 (m, 1H, H-3), 0.99 (d, *J*=6.6 Hz, 3H), 0.89 (t, *J*=7.4 Hz, 3H); ^{13}C NMR δ 41.0, 36.7, 27.5, 18.3, 11.2.

*(R)-1-Bromo-3-methylpentane (**8**) and (S)-1-Bromo-3-Methylpentane (**10**)* The known (*R*)-1-Bromo-3-methylpentane (**8**) was prepared from bromosulfonate **4** by a procedure (Nakamura and Mori 1999) that does not affect the chiral center (Fig. 2). ^{1}H NMR δ 3.50–3.36 (m, 2H, 1-H_2), 1.95–1.83 (m, 1H, H-2), 1.72–1.60 (m, 1H, H-2), 1.60–1.50 (m, 1H, H-3), 1.43–1.30 (m, 1H, H-4), 1.25–1.12 (m, 1H, H-4), 0.89 (d, *J*=6.3 Hz, 3H, 3-CH_3), 0.89 (t, *J*=7.2 Hz, 3H, 5-H_3). (*S*)-1-Bromo-3-methylpentane (**10**; Barrett et al. 1996) was synthesized in a similar fashion from bromosulfonate **9** [the (*R*)-enantiomer of **4**]. For direct comparison, (*S*)-1-bromo-3-methylpentane was also prepared from commercial (*S*)-3-methyl-1-pentanol (TCI America, Portland, OR, USA).

*(3R,7R,8S)-cis-7,8-Epoxy-3-methylnonadecane (**11**)* A solution of (*R*)-1-pentyl-3-methylmagnesium bromide [freshly prepared from 0.60 g (3.96 mmol) of (*R*)-1-bromo-3-

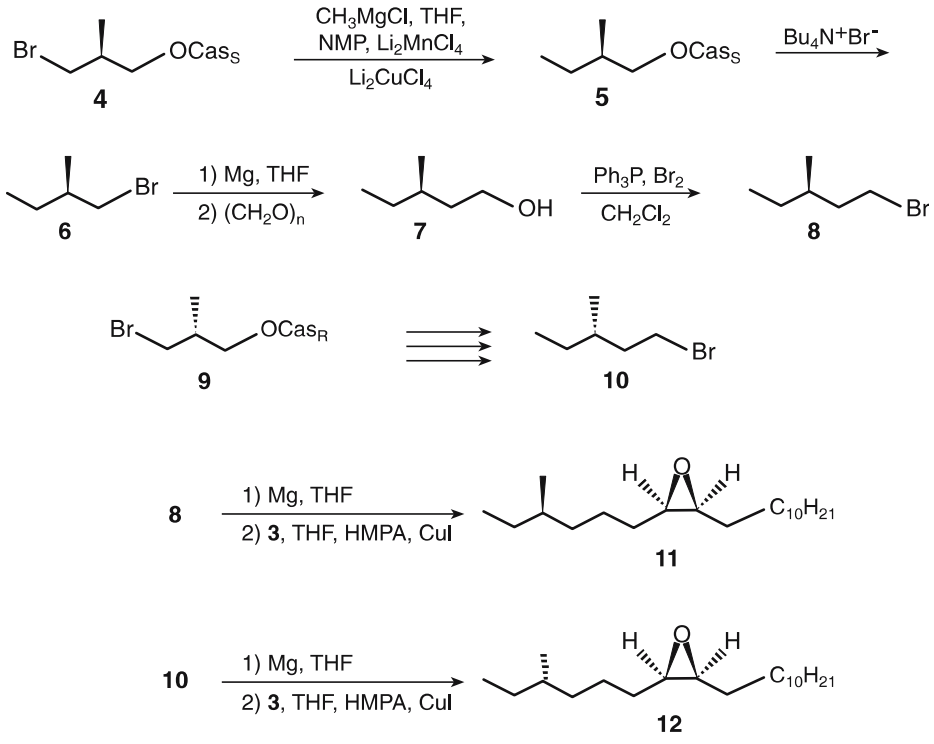


Fig. 2 Scheme for the enantioselective syntheses of (3*R*,7*R*,8*S*)- and (3*S*,7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane

methylpentane (**8**) and two equiv. of magnesium turnings by refluxing for 3 hr in 6 ml of degassed THF] was added dropwise to a stirred mixture of bromoepoxide **3** (372 mg, 1.28 mmol), CuI (0.073 g, 0.38 mmol), distilled HMPA (2.0 ml, 11.5 mmol), and THF (0.8 ml) at −23°. After 1 hr (no starting bromoepoxide **3** detectable by GC), the reaction mixture was warmed to 0°C and quenched with aq. NH_4Cl solution. The reaction products were extracted with hexane–ether (1:1), and the extracts were washed (water, brine) and dried (anhyd. $MgSO_4$). Solvents and low-boiling by-products were removed *in vacuo*. Flash chromatography (40 g SiO_2, 0–3% ether in hexane) yielded 290 mg (0.98 mmol) of pure epoxide **11** (77%); $[\alpha]_D^{20} = -4.57°$ (c 2.45, $CHCl_3$); IR (film) 2957, 2925, 2855, 1463, 1378, 1088, 909, 828 cm^{-1}; ^1H NMR δ 2.90 (m, 2H), 1.15–1.55 (4 m's, 29H), 0.86 (m, 9H); ^{13}C NMR δ 57.23, 57.21, 36.47, 34.34, 31.91, 29.63, 29.62, 29.56, 29.55(2), 29.42, 29.33, 28.16, 27.83, 26.59, 24.14, 22.67, 19.07, 14.09, 11.35. Anal. calcd. for $C_{20}H_{40}O$ (%): C, 81.00; H, 13.59; found C, 80.73; H 13.70.

*(3S,7R,8S)-cis-7,8-Epoxy-3-methylnonadecane (**12**)* Synthesis of compound **12** was analogous to that of **11**, starting with bromoepoxide **3** and (*S*)-1-bromo-3-methylpentane (**10**) derived from (*S*)-3-methyl-1-pentanol (TCI America). The final product **12** was separated by HPLC from unreacted starting material **3**. The yield (based on recovered **3**) was 40%. Spectral data of **12** were identical to those of **11** $[\alpha]_D^{20} = +6.40°$ (c 0.53, $CHCl_3$); for epoxide **12** derived from (*S*)-1-bromo-3-methylpentane that was produced from bromosulfonate **9**: $[\alpha]_D^{20} = +6.46°$ (c 0.65, $CHCl_3$); anal. calcd. for $C_{20}H_{40}O$ (%): C, 81.00; H, 13.59; found C, 81.30; H 13.35. Spectral data were identical to those of epoxide **11**.

Field Experiments Candidate pheromone components were field tested on the campus of Dr. Y. S. Parmar University of Horticulture and Forestry (Nauni, India; 30°51.52′N, 77° 10.18′E) and in oak stands of the Kandaghat Forest (30°57.27′N, 77°6.82′E) within 30 km of Solan (Himalach Pradesh, India). At 15- to 20-m intervals, sticky 2-l milk carton traps (Gray et al. 1984) were suspended 1.5 m above ground in randomized complete blocks. Traps were baited with a gray sleeve stopper (The West Co., Lionville, PA, USA) impregnated with test chemicals in HPLC-grade hexane.

Experiment 5 tested the two most abundant and antennal stimulatory components 2Me-7*Z*-18Hy and (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] singly at 50 μg each and in combination (50:25 μg) vs. unbaited traps. Because the unbaited control traps did not capture a single insect, they were excluded from follow-up experiments. Considering further that (+)-disparlure and 2Me-7*Z*-18Hy were attractive as single components in experiment 5 and apparently more attractive in combination, experiment 6 explored whether the attractiveness of 2Me-7*Z*-18Hy could be enhanced by either one or both enantiomers of disparlure. Based on synergistic attractiveness of 2Me-7*Z*-18Hy and (+)-disparlure in experiment 6, experiments 7 and 8 explored the optimal binary blend by testing components singly and at various ratios. Experiments 9 and 10 then investigated the potential role of minor candidate pheromone components. Experiment 9 tested whether one or both of 3*R*-Me-7*Z*-19Hy and 3*S*-Me-7*Z*-19Hy enhanced the attractiveness of 2Me-7*Z*-18Hy. The design of experiment 10 was based on the hypothesis that 3*R*-Me-7*Z*-19Hy and 3*S*-Me-7*Z*-19Hy may be effective only in combination with their corresponding *RS*-epoxides; thus, we tested all four components in combination for their effect on the binary blend of 2Me-7*Z*-18Hy and (+)-disparlure.

Trap catch data were subjected to nonparametric analyses of variance (Friedman's test) followed by comparison of means by the Scheffé test (Zar 1984; SAS/STAT 1988). In all analyses, α=0.05.

Results and Discussion

In mate acceptance studies with pairs of con- or heterospecific male and female *L. dispar* and *L. obfuscata*, male *L. dispar* and male *L. obfuscata* made copulatory attempts mainly when paired with a conspecific female (Table 1). Most male *L. obfuscata* paired with a female *L. dispar* also attempted copulation, suggesting that (+)-disparlure produced by female *L. dispar* as a single sex pheromone component was attractive and thus might be a pheromone component also of female *L. obfuscata.* These results further suggested that other volatile constituents present in, or released from, pheromone glands of female *L. dispar* [*cis*-7,8-epoxy-2-methylheptadecane; *cis*-7,8-epoxy-3-methylnonadecane; (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadec-17-ene (Gries et al. 1996, 2005b)] did not antagonize male *L. obfuscata*. Conversely, the few copulatory attempts male *L. dispar* made when paired with female *L. obfuscata* (Table 1) suggested that female *L. obfuscata* emit one or more volatile components antagonistic to male *L. dispar*. We hypothesized that these components were part of the *L. obfuscata* sex pheromone blend and initiated pheromone analysis.

GC–EAD analyses of pheromone gland extract from female *L. obfuscata* revealed eight components that consistently elicited responses from male antennae (Fig. 3). The two most abundant components **C** and **G**, which also elicited the strongest responses from antennae, had GC retention and mass spectrometric characteristics entirely consistent with (*Z*)-2-methyloctadec-7-ene (**C**) and the corresponding epoxide disparlure (**G**). Component **H** had retention indices (RI; relative to alkane standards; Van Den Dool and Kratz 1963) on three GC columns (DB-5: 2140; DB-23: 2459; DB-210: 2422) consistent with those of *cis*-7,8-epoxy-3-methylnonadecane, a trace component in pheromone gland extracts of *L. dispar* (Gries et al.

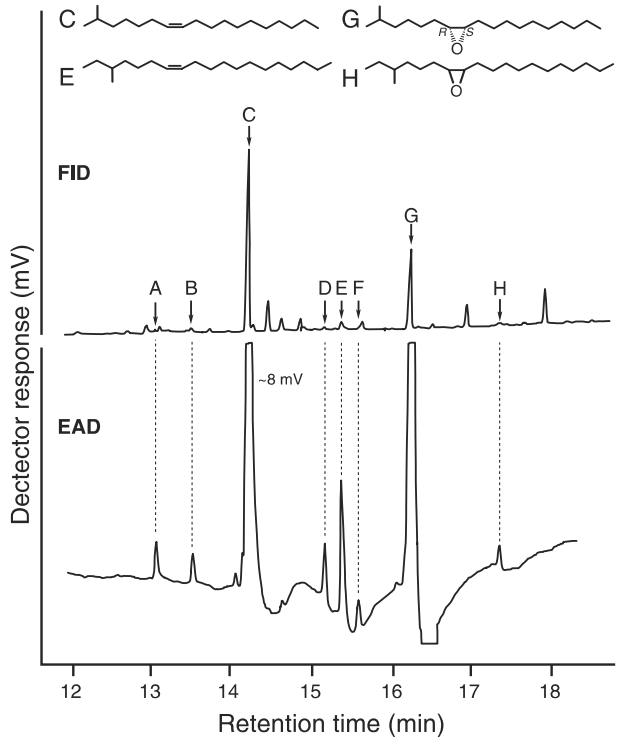


Fig. 3 Representative recording (*N*=6) of flame ionization detector (*FID*) and electroantennographic detector (*EAD*: male *L. obfuscata* antenna) responses to one female equivalent of pheromone gland extract of *L. obfuscata*. Compound identities: *A* unknown; *B* unknown; *C* (*Z*)-2-methyloctadec-7-ene [2Me-7*Z*-18Hy]; D unknown; E (3*R*,7*Z*)- and/or (3*S*,7*Z*)-3-methylnonadecene [3*R*-Me-7*Z*-19Hy and/or 3*S*-Me-7*Z*-19Hy]; *F* unknown; *G* (7*R*,8*S*)-*cis*-7,8-epoxy-2-methyloctadecane [=(+)-disparlure]; *H* (3*R*,7*R*,8*S*)- and/or (3*S*,7*R*,8*S*)-*cis*-7,8-epoxy-3-methylnonadecane [3*R*-Me-7*R*,8*S*-epo-19Hy and/or 3*S*-Me-7*R*,8*S*-epo-19Hy]

1996). Structural assignment of component **H** was indirectly supported by the presence of the corresponding hydrocarbon (*Z*)-3-methylnonadec-7-ene (**E**), which had a mass spectrum and retention indices (DB-5, 1954; DB-23, 1988; DB-210, 1971) entirely consistent on all three columns with those of an authentic standard. Components **A**, **B**, **D**, and **F** occurred in quantities well below FID detection threshold, and their structures are not yet known.

The identification of 2Me-7*Z*-18Hy in pheromone gland extracts of female *L. obfuscata* explains the relatively few copulatory attempts male *L. dispar* made when paired with a female *L. obfuscata* (Table 1). 2Me-7Z-18Hy is a pheromone component of sympatric *L. dispar* congeners [*Lymantria monacha* in Europe (Grant et al. 1996; Gries et al. 1996) and Japan (Gries et al. 2001); *L. fumida* and *L. lucescens* in Japan (Schaefer et al. 1999; Gries et al. 2002b)] which, when added to (+)-disparlure baits, strongly reduces attraction of male *L. dispar* in Europe (Grant et al. 1996; Gries et al. 1996), North America (Cardé et al. 1973) and Asia (GG and PWS, unpublished).

Epoxide **3** was prepared from commercially available epoxide **1** (Gries et al. 2005b; Fig. 1). The conversion of **1** to **2** proceeded in 79% yield. A more convenient procedure, omitting Li_2MnCl_4 and using HMPA and CuI in place of NMP and Li_2CuCl_4, afforded **2** in 67% yield. In the conversion of **2** to **3**, elevated temperature (67°C) is essential to achieve complete reaction in minimal time; the presence of acid/water must be avoided to maximize the reaction rate and minimize product isomerization to a *trans*-epimer of **3**. Byproduct $Bu_4N^+Cas_sO^-$ is easily recovered from the aqueous phase from the reaction workup by evaporation of the water. Synthesis routes to target epoxides **11** and **12** are outlined in Fig. 2. Access to commercially unavailable (*R*)-1-bromo-2-methylbutane (**6**) was gained by chemoselective coupling of CH_3MnCl (Cahiez and Marquais 1993) with bromoalkyl camphorsulphonate (casylate) **4** (crystalline solid; >99% pure; R. Hahn, F. B. Cook, and M.-Y. He, unpublished data) to give the casylate ester of (*R*)-2-methyl-1-butanol (**5**; 94% yield), followed by displacement of casylate by bromide (95%). Bromide **6** and its enantiomer derived from bromo-casylate **9** were converted (Pino et al. 1959 and references therein; Schaefer et al. 1973 and references therein; Bestmann et al. 1998) to known **8** and **10**, respectively. Grignard reagents from the latter two compounds were then coupled with epoxide **3**, to afford **11** and **12**. Additional quantities of 3*S*-Me-7*R*,8*S*-epo-19Hy (**12**) were obtained from commercially available (*S*)-3-methyl-1-pentanol (the enantiomer of alcohol **7**) via bromide **10** (Fig. 2). NMR and IR spectral data and optical rotation values of the final epoxides **12** obtained from the bromides **10** obtained by the two different routes were identical. Spectral data for 3*R*-Me-7*R*,8*S*-epo-19Hy (**11**) are identical to those of its diastereomer **12**. Optical rotation values differed considerably.

In experiment 5, (+)-disparlure and 2Me-7*Z*-18Hy as single-component lures attracted male *L. obfuscata* (Fig. 4). Both components in combination appeared more attractive, but results were not statistically analyzed because many traps were damaged or removed by monkeys. In experiment 6, (+)-disparlure significantly enhanced attractiveness of 2Me-7*Z*-18Hy, whereas (−)-disparlure had neither synergistic nor antagonistic effects (Fig. 4). In experiments 7 and 8, combinations of (+)-disparlure and 2Me-7*Z*-18Hy at blend ratios of 0.5:1, 5:1, 1:2, and 1:10 were of similar effectiveness and more effective than either component alone in attracting male *L. obfuscata* (Fig. 5). The attractiveness of 2Me-7*Z*-18Hy in experiment 9, or 2Me-7*Z*-18Hy plus (+)-disparlure in experiment 10, was not affected by the addition of other candidate pheromone components (Fig. 6).

Laboratory and field data support the conclusion that the sex pheromone of *L. obfuscata* comprises a blend of two synergistic components: 2Me-7*Z*-18Hy and (+)-disparlure. The blend is dissimilar to the single-component sex pheromone (+)-disparlure in populations of European and Asian *L. dispar*. Male *L. obfuscata* and male *L. dispar* also differ in their response to (−)-disparlure. (−)-Disparlure strongly antagonizes attraction of male *L. dispar* to pheromone sources

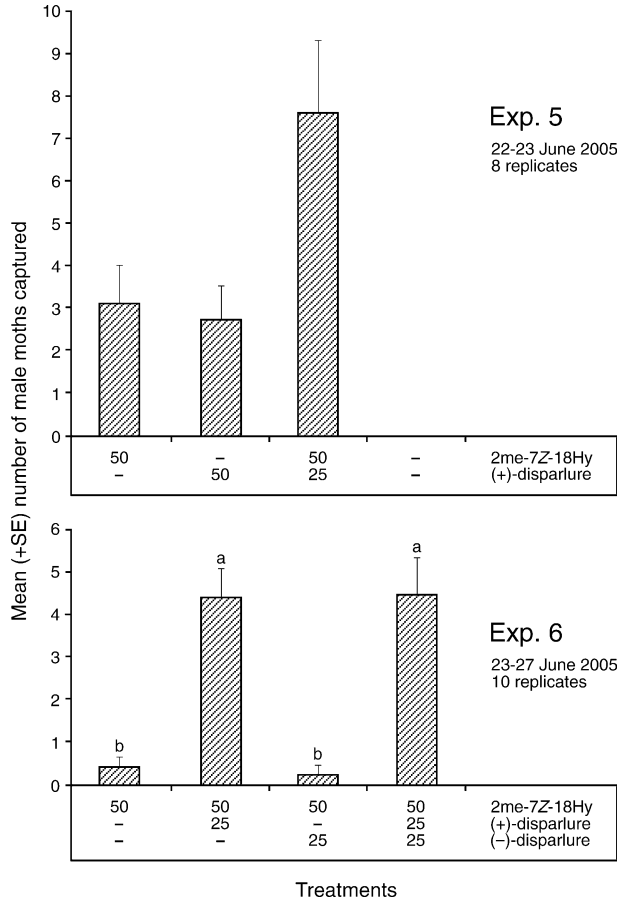


Fig. 4 Mean (+SE) number of male *L. obfuscata* captured per experimental period in experiment 5 (30°51.52′N, 77°10.18′E) and experiment 6 (coordinate data lost, site on road to Pomology research area on campus of Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, India) in sticky traps baited with candidate pheromone components. Bars in experiment 6 with different letter superscripts are significantly different; nonparametric analyses of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test), $P<0.05$ (SAS/STAT 1988). Compound nomenclature as in caption of Fig. 3

(Klimetzek et al. 1976; Cardé et al. 1977a, b; Miller et al. 1977; Plimmer et al. 1977) but does not affect the behavior of male *L. obfuscata.* The dissimilar sex pheromones of *L. obfuscata* and *L. dispar* and the contrasting response to (−)-disparlure both support taxonomic distinction of *L. obfuscata* and *L. dispar*.

If we consider the presence of congeners a biological factor that shapes the sexual communication signal of individual species (Linn and Roelofs 1995), then the dissimilar sex pheromones of *L. obfuscata* and *L. dispar* could be explained by the contrasting composition of the lymantriid communities they inhabit. None of the congeners *L. monacha*, *L. fumida*, and *L. lucescens* that produce 2Me-Z7-18Hy as a sex pheromone component, with strong antagonism of heterospecific male *L. dispar*, is present in the *L. obfuscata* community. Thus, 2Me-7*Z*-18Hy can be part of the *L. obfuscata* sex pheromone. Similarly, *L. monacha* in sympatry with *L. dispar* purportedly produces (−)-disparlure (Hansen 1984), which enhances the specificity of the communication channel and reduces cross-attraction of male *L. dispar* (Klimetzek et al. 1976; Cardé et al. 1977a, b; Miller et al. 1977; Plimmer et al. 1977). With *L. monacha* absent from the *L. obfuscata* community, male *L. obfuscata* did not evolve to recognize and be antagonized by (−)-disparlure (Fig. 4).

It would now be intriguing to study potential divergence of the *L. obfuscata* sex pheromone in lymantriid communities of different composition, particularly in the Pamir Mountains of Tajikistan where *L. obfuscata* and *L. dispar* may co-occur, as proposed by Schintlmeister (2004).

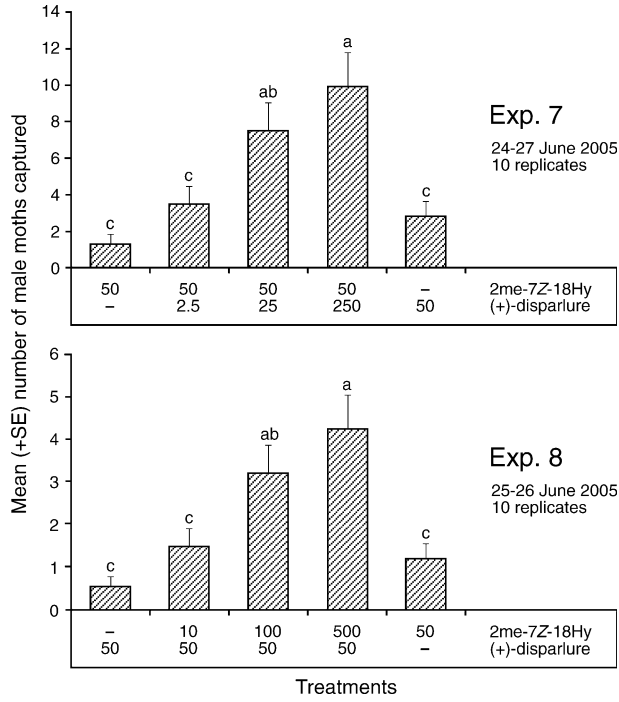


Fig. 5 Mean (+SE) number of male *L. obfuscata* captured per experimental period in experiment 7 (30°57.29′N, 77°6.82′E) and experiment 8 (30°56.98′N, 77°7.27′E) in sticky traps baited with candidate pheromone components singly and in binary combination at different ratios. Bars in each experiment with different letter superscripts are significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test), $P<0.05$ (SAS/STAT 1988). Nomenclature as in caption of Fig. 3

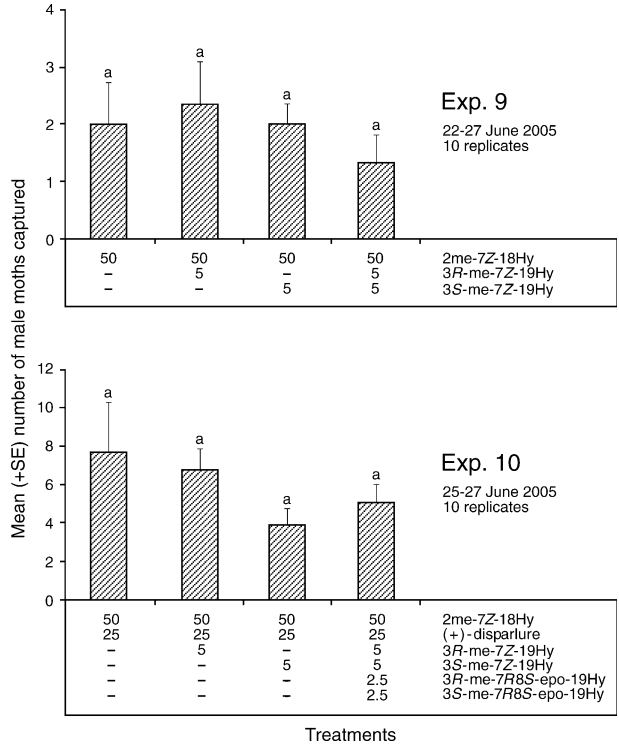


Fig. 6 Mean (+SE) number of male *L. obfuscata* captured per experimental period in experiment 9 (30°51.76′N, 77°10.28′E) and in experiment 10 (near 30° 57.3′N, 77°6.8′E) in sticky traps baited with the binary blend of (+)-disparlure and (7*Z*)-2-methyloctadec-7-ene alone or in combination with other candidate pheromone components. *Bars* in each experiment with the same letter superscript are not significantly different; nonparametric analysis of variance (Friedman's test) followed by comparison of means (Scheffé test), $P<0.05$ (SAS/STAT 1988). Compound nomenclature as in caption of Fig. 3

Acknowledgments We thank Dharam Pal and Ravinder Sharma for field assistance, Girish Sharma and Amit Nath for guidance on use of suitable trapping sites, Devinder Singh for local transportation, Sanjeev Thakur for permission to run field experiments in the Kandaghat Forest Range, Gagandeep S. Hehar for logistical help, T. D. Verma and Prem Raj Gupta for information on *L. obfuscata* biology and behavior, Susan Barth for moth rearing, Mei-Keng Yang for elemental analyses, Stephen Takács for some statistical analysis, Eberhard Kiehlmann for review of the manuscript, and Jocelyn Millar and two anonymous reviewers for constructive comments. The research was supported by a Natural Sciences and Engineering Research Council of Canada—Industrial Research Chair to G.G., with Pherotech International Inc., SC Johnson Canada, and Global Forest Science (GF-18-2007-226; GF-18-2007-227) as industrial sponsors. Insects were maintained in SFU's Global Forest Quarantine Facility, construction of which was completed with financial support from Global Forest Science.

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of head and mandibles (for a review see Deligne et al. 1981; Prestwich 1984); (2) evolution of the frontal gland as a unique chemical defense tool in the advanced termite families Serritermitidae, Rhinotermitidae, and Termitidae (Costa-Leonardo and Kitayama 1991); and (3) related behavioral and defensive strategies (Pasteels and Bordereau 1998). Frontal gland defensive secretions of termite soldiers may contain different chemical components, including mono-, sesqui-, and diterpenoid hydrocarbons, alcohols, ethers, ketoaldehydes, fatty acids, macrocyclic lactones, etc. (Prestwich 1978, 1984; Prestwich et al. 1978, 1980; Prestwich and Collins 1980, 1981a, b; Naya et al. 1982; Pasteels and Bordereau 1998; Chen et al. 1999). The deterrent property of such a secretion is often enhanced by the formation of a glue-like material after release that immobilizes enemies (Deligne et al. 1981).

Apart from its defensive role, frontal gland substances can be involved in alarm communication, in more advanced families (e.g., Eisner et al. 1976; Deligne et al. 1981; Pasteels and Bordereau 1998). The role of individual compounds of the secretion in the elicitation of alarm has been confirmed only in a few cases. α-Pinene and limonene in *Nasutitermes rippertii*, 3-carene and limonene in *Nasutitermes costalis* (Vrkoč et al. 1978), and α-pinene in *Nasutitermes princeps* (Roisin et al. 1990) have all been shown to be alarm pheromone components.

The 17 species of the genus *Prorhinotermes* (Isoptera: Rhinotermitidae) form the subfamily Prorhinotermitinae (Quennedey and Deligne 1975). This subfamily, despite many advanced characteristics, also displays several primitive traits, such as a linear ontogeny lacking a true worker caste (Roisin 1988) and a nesting habit without a well-defined centralized nest. These species can be classified as single-site nesters, with colonies located in dead logs and stumps, usually in close vicinity of water sources (e.g., Banks and Snyder 1920; Shellman-Reeve 1997). The distribution of *Prorhinotermes* species is cosmotropical with maximum diversity in Southeast Asia; the species usually live on islands and are found rarely on the mainland. The island distribution is likely a consequence of the ability of these species to cross the open ocean in floating logs or coconuts (Emerson 1955).

In colonies of *Prorhinotermes*, soldiers are abundant, often comprising more than 10% of the population. Soldiers defend the colony interior as well as foraging groups and possess, in addition to slashing falcate mandibles, a voluminous frontal gland. The reservoir of this gland extends over the whole abdomen and can be observed as a transparent sac (Šobotník et al. 2004). (*E*)-1-Nitropentadec-1-ene is the major defensive compound of *Prorhinotermes simplex* (Hagen), *Prorhinotermes inopinatus* Silvestri, and *Prorhinotermes flavus*, the only *Prorhinotermes* soldiers analyzed to date. This chemical is present in high amounts in the body of soldiers (up to 370 μg/individual, ca. one tenth of the average soldier body weight) (Vrkoč and Ubik 1974; Chuah et al. 1990; Kuldová et al. 1999; Šobotník et al. 2004). (3*Z*,6*E*)-α-Farnesene has also been identified in the frontal gland secretion of *P. simplex* soldiers (Naya et al. 1982). It is well accepted that the volatile compounds from this gland serve as an alarm pheromone (Moore 1969).

In this study, we compared frontal gland contents of *Prorhinotermes* species from Neotropical (*P. simplex*), Malagasy (*P. canalifrons* Sjöstedt), and Papuan (*P. inopinatus*) regions to ascertain knowledge about the qualitative and quantitative chemodiversity of this gland secretion.

Methods and Materials

General

Most chemicals were obtained from Sigma-Aldrich or Fluka. (3*Z*,6*E*)-α-Farnesene and (*E*)-1-nitropentadec-1-ene were synthesized earlier in our laboratory (Svatoš and Attygalle

1997; Kuldová et al. 1999). (*E*)-1-Nitroheptadec-1-ene was synthesized from hexadecanal, analogous to the synthesis of (*E*)-1-nitropentadec-1-ene (Kuldová et al. 1999). *Ocimum basilicum* essential oil was purchased from Essential Oil University and lime essential oil from Aldrich.

Quantitative gas chromatography-mass spectrometry (GC-MS) analysis was carried out on a Varian Saturn 2000R ion-trap GC-MS instrument with electron ionization (70 eV). A DB-5 ms fused silica capillary column (30 m×0.25 mm, film thickness 0.25 μm, J & W Scientific) was used for the analyses with the following temperature program: 70°C for 2 min, increased to 200°C at a rate of 20°C per minute, and then to 320°C at 10°C per minute. Splitless injection was used with helium as a carrier gas. Each sample was analyzed ×3, and mean values were calculated for the integrated areas by using calibration curves. The calibration curves were calculated with solutions of different concentration ratios of (3*Z*,6*E*)-α-farnesene, and (*E*)-1-nitropentadec-1-ene to 1-bromodecane.

For identification of compounds, EI mass spectra (70 eV) were recorded on a Fisons MD800 quadrupole MS coupled to a Finnigan Focus GC (Thermo) fitted with a ZB-5 ms capillary column (30 m×0.25 mm, film thickness 0.25 μm, Zebron Phenomenex) that employed conditions as above. Identification was generally based on fragmentation pattern together with comparison of retention indices. However, for the various sesquiterpenes, as well as (*E*)-1-nitropentadec-1-ene and (*E*)-1-nitroheptadec-1-ene, coinjection with standards was also carried out. *Ocimum basilicum* essential oil, containing *trans*-β-bergamotene (Bini et al. 2000), and lime essential oil, containing (*Z*)-γ-bisabolene (Joulain and Koenig 1998), were employed as a source of standards.

Retention indices on both ZB-5 and DB-wax (30 m×0.25 mm, film thickness 0.25 μm, J & W Scientific) columns were obtained by using a mixture of *n*-alkanes (C8–C22) as standards, and methane as an unretained compound. For the ZB-5 column, a temperature program of 60 to 240°C at 3°C per minute, then to 320°C at 20°C per minute, was used, while the DB-wax was kept isothermally at 165°C.

The Fourier transform infrared (FT-IR) spectra were obtained by using a Finnigan GCQ GC interfaced with a Bruker Equinox 55 system (FT-IR resolution, 8 cm^{-1}; light pipe temperature, 200°C), equipped with a DB-5 capillary column (30 m×0.32 mm, film thickness 0.25 μm, J & W Scientific) and an oven temperature program of 70°C for 2 min, increased to 250°C at 10°C per minute. Injection was splitless, and He was the carrier gas.

Insects

Soldiers were removed from mature laboratory colonies of *P. canalifrons* (collected in Réunion in 2001), *P. inopinatus* (collected at Kau Wildlife Area, Madang Province, Papua New Guinea, in 2000), and *P. simplex* (collected in Soroa, Piñar del Rio, Cuba, in 1964). All colonies were kept in permanent darkness at 26±1°C with elevated humidity (>85%). Soldiers that molted from late pseudergate instars were chosen for the analysis. These individuals do not differ greatly in their outer anatomy or size among the three species (Fig. 1).

Extracts of Soldiers

Immediately after removal from the colony, soldiers were frozen at −20°C for 5 min and dissected transversely at the third abdominal segment to open the gland. 1-Bromodecane in hexane (50 μl; 0.554 mg ml^{-1} for *P. canalifrons*, 0.576 mg ml^{-1} for *P. inopinatus* and *P. simplex*) was used to extract single individuals. Ten samples each of *P. canalifrons* and

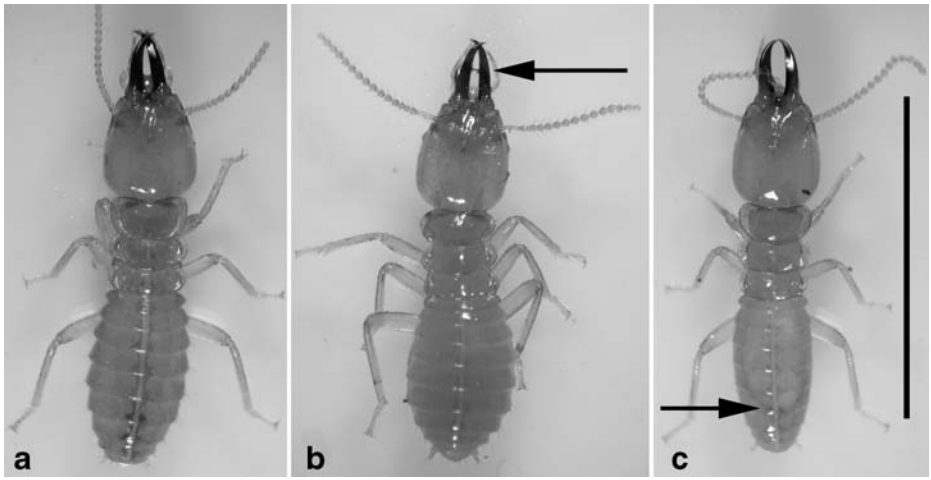


Fig. 1 Soldiers of three *Prorhinotermes* species: **a** *P. canalifrons*; **b** *P. inopinatus* (the released frontal gland secretion between the mandibles is marked with an *arrow*); **c** *P. simplex* (the frontal gland reservoir ending in the seventh abdominal segment is marked with an *arrow*). The *bar* represents 5 mm

P. inopinatus, and three samples of *P. simplex* were prepared. The samples were shaken for 10 min and the extracts were separated from the termites and then stored at −20°C.

Additionally, an extract of 10 *P. canalifrons* soldiers was prepared to establish retention indices of the compounds on a polar column. To confirm the origin of the sesquiterpenes and nitroalkenes, the contents of a *P. simplex* soldier frontal gland were carefully removed with a microcapillary by piercing the body wall at the third abdominal segment and then analyzed by GC-MS.

Results and Discussion

GC-MS analysis of the three species revealed large quantities of (*E*)-1-nitropentadec-1-ene, along with much lower amounts of the homologs, (*E*)-1-nitroheptadec-1-ene, 1-nitrotridec-1-ene, and trace amounts of 1-nitrotetradec-1-ene, 1-nitrohexadec-1-ene, nitropentadecadiene, and nitroheptadecadiene. The composition and relative ratios of the nitroalkenes were similar across the three species. The much lower amounts of the nitroalkenes with an even number of carbon atoms, in comparison to those with an odd number, follows the pattern for hydrocarbons and their derivatives in insect cuticle (Blomquist and Dillwith 1985).

All the nitroalkenes from the frontal gland extracts gave mass spectra similar to that of (*E*)-1-nitropentadec-1-ene with diagnostic ions of $M^{+\bullet}$, $[M–OH^{\bullet}]^{+}$, and $[M–OH^{\bullet}–H_2O]^{+}$ (Table 1), together with *m/z* 30 and 46, corresponding to $[NO]^{+}$, and $[NO_2]^{+}$, respectively. In the mass spectrum of all of the nitro-olefins, a peak at *m/z* 87 was found and assigned to $[C_3H_5NO_2]^{+}$, which originates from α-cleavage of 1-nitro-olefins with the conjugated double bond $RCH{=}CHNO_2$. This assignment of *m/z* 87 was confirmed by analysis of the (*E*)-1-nitropentadec-1-ene standard, which also contained (*E*)-1-nitropentadec-2-ene impurity. No *m/z* 87 was present in the mass spectrum of the latter compound, which eluted prior to nitropentadec-1-ene on the ZB-5 column and which otherwise gave a similar fragmentation pattern. The identity of (*E*)-1-nitropentadec-2-ene was confirmed by GC/FT-IR. In alkenes, the position of the nitro group (a strong electron-withdrawing group), with respect to the

Table 1 Analytical data of nitro compounds from frontal gland of *P. canalifrons* soldiers

Compound	*m/z*: $M^{+\bullet}$, $[M–OH^{\bullet}]^+$, $[M–OH^{\bullet}–H_2O]^+$	RI on ZB-5
Nitrotridecane	229, 212, 194	1,762
1-Nitrotridec-1-ene	227, 210, 192	1,808
1-Nitrotetradec-1-ene	241, 224, 206	1,913
Nitropentadecane	257, 240, 222	1,976
Nitropentadecadiene	253, 236, 218	1,984
(*E*)-1-Nitropentadec-1-ene	255, 238, 220	2,019
1-Nitrohexadec-1-ene	269, 252, 234	2,092
Nitroheptadecadiene	281, 264, 246	2,193
(*E*)-1-Nitroheptadec-1-ene	283, 266, 248	2,222

RI = retention index

double bond, determines the wavelength of the C–H asymmetrical stretching, C=C stretching, and C–H wagging peaks in the infrared spectrum, thus allowing determination of nitroalkene configuration (Brown 1955). Peaks at 3,043, 1,668, and 968 cm^{-1} in the gas-phase FT-IR spectrum of this compound shifted in comparison with the values found for (*E*)-1-nitropentadec-1-ene (Table 2), indicating the presence of the $-CH=CHCH_2NO_2$ moiety in *trans* configuration.

An attempt to determine the carbon–carbon double bond position in the nitroalkenes from soldiers by chemical ionization ion-trap MS using acetonitrile failed to form any adducts, further supporting the presence of the $-CH=CHNO_2$ moiety in these chemicals. In this method, the ion $[C_3H_4N]^+$, originating from fragmentation of acetonitrile, reacts with a C=C double bond, indicating the position of the double bond (Oldham and Svatoš 1999). However, the $-CH=CHNO_2$ moiety did not react with $[C_3H_4N]^+$ due to the strong electron withdrawing effect of the nitro group conjugated with the carbon–carbon double bond.

The amounts of nitropentadecene in the soldier frontal gland secretions were fairly similar among the three *Prorhinotermes* species, reaching up to 300 μg/individual (Table 3). Apart from the nitroalkenes, we also detected two nitroalkanes (Table 1) at similar amounts in all samples. It has previously been shown that *P. simplex* workers reduce (*E*)-1-nitropentadec-1-ene, secreted by colony soldiers, to 1-nitropentadecane using a specific oxidoreductase (Spanton and Prestwich 1981), thereby detoxifying the defensive compound. We believe that nitrotridecane and nitropentadecane found in our extracts may be detoxification products in soldiers.

Although the nitroalkene content in the frontal gland was similar across the three species, the sesquiterpene hydrocarbon content differed considerably. Soldiers of *P. simplex* contained (3*Z*,6*E*)-α-farnesene, with no other α-farnesene or sesquiterpene isomers detected. *P. inopinatus* soldiers contained two geometric isomers of α-farnesene, (3*Z*,6*E*) and

Table 2 Diagnostic gas-phase FT-IR absorption bands of 1-nitroalkenes

Compound	H–C str, C=C str, H–C wag (cm^{-1})
(*E*)-1-nitropentadec-1-ene	3,108, 1,658, 960
Ref–*trans* $RCH=CHNO_2$[a]	3,106–3,049, 1,656–1,647, 962–958
(*E*)-1-nitropentadec-2-ene	3,043, 1,668, 968
Ref–*trans* $RCH=CHCH_2NO_2$[a]	3,049–3,012, 1,678–1,664, 970–962

[a] Brown 1955

Table 3 Quantity (range, median, and – in bold – mean value with standard sample deviation) of main components of frontal gland secretion in soldiers of three *Prorhinotermes* species

	P. canalifrons	*P. inopinatus*	*P. simplex*
	N=10	*N*=10	*N*=3
(3*Z*,6*E*)-α-Farnesene (μg/individual)	0.15–0.76[a]	0.5–1.9	25–57
	0.52[a]	1.4	36
	0.49±0.20[a]	**1.5±0.7**	**39±13**
(3*E*,6*E*)-α-Farnesene (μg/individual)	1.0–3.8	0.5–1.0	Not detected
	1.55	0.6	
	1.8±0.8	**0.7±0.1**	
(*E*)-1-Nitropentadec-1-ene (μg/individual)	30–285	115–310	225–387
	153	197	269
	152±89	**207±60**	**294±68**

[a] For six individuals only: in three individuals, the level of (3*Z*,6*E*)-α-farnesene was too low for a reliable quantification and in the other it has not been detected

(3*E*,6*E*), while *P. canalifrons* soldiers contained the same two α-farnesene isomers along with smaller amounts of *trans*-β-bergamotene and (*Z*)-γ-bisabolene (Table 4). *P. canalifrons* and *P. inopinatus* differed significantly ($P<10^{-3}$, Mann–Whitney *U* test) in the relative amounts of the two α-farnesenes (Table 3, Fig. 2). The main sesquiterpene in *P. inopinatus* is (3*Z*,6*E*)-α-farnesene with lower amounts of the (3*E*,6*E*) isomer. However, in *P. canalifrons*, the (3*E*,6*E*) isomer was more abundant. The quantity of (3*Z*,6*E*)-α-farnesene in *P. simplex* soldiers was much higher than in soldiers of the other two species (Table 3), in agreement with our earlier report (Šobotník et al. 2004) .

Table 4 Analytical data of sesquiterpene hydrocarbons from frontal gland secretion of *P. canalifrons* soldiers

Compound	Mass spectrum *m/z* (%)	RI on ZB-5	Ref–RI on DB-5[a,b]	Ref–RI on DB-1[c]	RI on DB-wax[d]	Ref–RI on Carbowax[e,f,g]
Trans-β-bergamotene	204 (2), 93 (47), 91 (35), 79 (36), 69 (100), 67 (29), 55 (24), 41 (69), 39 (21)	1,479		1,480	1,717	
(3*Z*,6*E*)-α-Farnesene	204 (1), 119 (60), 107 (35), 93 (100), 91 (49), 79 (50), 69 (57), 55 (54), 41 (75)	1,488		1,480	1,721	
(3*E*,6*E*)-α-Farnesene	204 (2), 119 (32), 107 (44), 93 (100), 91 (46), 79 (55), 69 (58), 55 (54), 41 (94)	1,502	1,505	1,498	1,745	1,756
(*Z*)-γ-Bisabolene	204 (10), 119 (55), 107 (75), 93 (100), 91 (50), 69 (35), 55 (39), 43 (22), 41 (57)	1,506	1,514	1,505	1,762	

RI = retention index

[a] The ZB-5 and DB-5 columns contain stationary phases of the same composition

[b] Adams 2007

[c] Joulain and Koenig 1998

[d] Isothermal (165°C)

[e] Isothermal (130°C)

[f] Davies 1990

[g] The DB-wax and Carbowax columns contain stationary phases of the same composition

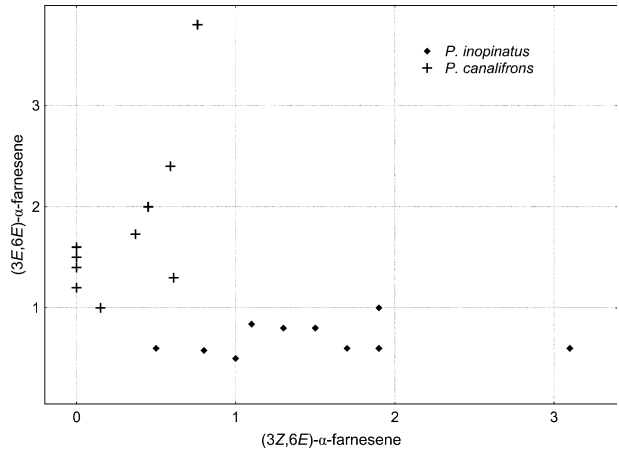


Fig. 2 Quantities (μg/individual) of α-farnesene isomers from the frontal gland of individuals of *P. inopinatus* and *P. canalifrons* soldiers

In comparison to the closely related *Coptotermes* genus, in which the defensive secretion of lactones forms a glue-like material after release (Deligne et al. 1981), the *Prorhinotermes* secretion remains fully liquid. The large amounts of chemicals found in an individual soldier in our analyses are probably sufficient to defeat numerous intruders; for example, 10 μg of nitropentadecene is the LD_{50} for the house fly *Musca domestica* (Kuldová et al. 1999). In addition, the relatively large percentage (10%) of soldiers in a *Prorhinotermes* colony means that large amounts of defensive compounds are available for repelling numerous intruders or even relatively large predators from the colony. Although this may provide good protection for *Prorhinotermes* colonies, it comes at a relatively high price: the use of relatively large amounts of rare nitrogen.

Acknowledgements We are grateful to Christian Bordereau and Alain Robert for providing us with the *P. canalifrons* colony. The *P. inopinatus* material was obtained thanks to a project funded by the US National Science Foundation (DEB-02-11591). We kindly acknowledge Anna-Karin Borg-Karlson for the gift of pure (3*E*,6*E*)-α-farnesene and Erin Walsh for the language corrections. Financial support was provided by the Grant Agency of the Academy of Sciences of the Czech Republic (A600550614), and the Z4 055 0506 project realized in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague.

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Introduction

Egg hatching on the Caribbean spiny lobster, *Panulirus argus*, is a highly synchronous event occurring before the ebbing tide, which assists the larvae in being transported by currents to offshore nursery areas (Yeung and Lee 2002). This timing is critical as it ensures that larvae are released at times in which transport is optimal as well as at times that minimize the threat of predation and exposure of larvae to adverse conditions (Forward 1987; Morgan 1995).

Synchrony of larval release among marine crustaceans is mediated by chemical-based communication between the hatching eggs and the ovigerous female (Rittschof et al. 1985, 1989, 1990a, b; Forward et al. 1987; De Vries and Forward 1991a; De Vries et al. 1991; Ziegler and Forward 2007). Pheromones originating from the hatching embryos induce the ovigerous female to perform stereotypical larval release behaviors (e.g., Ennis 1973, 1975; Branford 1978; Moller and Branford 1979; DeCoursey 1983; De Vries and Forward 1991a; Ziegler and Forward 2007). Among spiny lobsters, the female lobster uses her walking legs to probe her egg mass while she rapidly flexes and extends her abdomen, all the while actively beating the pleopods back and forth. During larval release, this vigorous "pleopod pumping" behavior assists in the egg hatching process by compressing the egg mass. Compression of the egg mass helps to break open egg membranes and free the larvae, which then swim into the water column. As more eggs hatch, more pheromone is released, causing the female to pump harder. This positive feedback system leads to a synchronous release of larvae (Forward 1987; Tankersley et al. 2002; Ziegler and Forward 2007).

Previous experiments suggest that chemical cues mediate these behaviors among spiny lobsters. Pleopod pumping activity of ovigerous females with late-stage embryos increases when they are exposed to hatch water (the medium into which larvae have been released; Ziegler and Forward 2007). Solutions of homogenized late-stage embryos and homogenized egg cases also induce larval release behaviors in lobsters with late-stage embryos. These observations suggest that a substance(s) is released at the time of hatching that acts to induce stereotypical pleopod pumping behaviors of the female.

Peptides are effective communication molecules in the marine environment because of their high solubility, high specificity, short half-life, and high signal-to-noise ratio (Rittschof et al. 1992; Pettis et al. 1993; Atema 1995; Decho et al. 1998; Rittschof and Cohen 2004). Small peptides originating from the proteolysis of egg cases may be the compounds responsible for coordinating synchronous release of larvae, as crabs display similar larval release pumping behaviors when exposed to solutions containing specific peptides (Rittschof et al. 1985, 1989; Forward et al. 1987). For the crab *Rhithropanopeus harrisii*, the developing embryos control the exact time of hatching, whereas the female controls the synchrony of hatching (Forward and Lohmann 1983). Ovigerous females exposed to partially identified native peptides or to pheromone analogs will initiate the pumping response, a behavior used to measure pheromone activity (Forward et al. 1987; Rittschof et al. 1989; Pettis et al. 1993).

We hypothesize that ovigerous spiny lobsters will respond to the same peptides that produce a larval release pumping response in brachyuran crabs (Forward et al. 1987; Pettis et al. 1993; Rittschof 1993). We used the stereotypic larval release behaviors of ovigerous spiny lobsters as a biological assay to investigate the chemistry of the substances released upon egg hatching. Measurements of pleopod pumping frequencies in response to various model peptides at several concentrations were used to determine which chemical cues are used by lobsters. These substances are collectively termed "pumping pheromones". We tested a logical series of di- and tripeptides and two oligopeptides: bradykinin, a barnacle settlement pheromone mimic recently implicated as a larval release pheromone (Rittschof

and Cohen 2004); as well as its complement des–Arg^9–bradykinin, which lacks arginine (Arg) at the basic carboxyl terminus. These compounds were chosen based upon the compositional analysis of partially purified native pheromones from previous studies on brachyuran crabs (e.g., Forward et al. 1987; Rittschof et al. 1985, 1989). No peptides were extracted from the lobsters; rather, we evaluated these logical synthetic candidates for activity. We demonstrate that pleopod pumping responses are evoked by peptides, which have a neutral amino acid at the amino terminus and a basic amino acid at the carboxy terminus, suggesting that specific peptides mimic or act as crustacean pheromones. According to the model for egg hatching, trypsin-like enzymes from hatching eggs would break down the egg membrane and cleave these peptides that are released in the water. The parent female senses the chemical, which stimulates her to pump her pleopods, and thus, results in the synchronous release of larvae.

Methods and Materials

Collection and Maintenance of Spiny Lobsters Ovigerous *P. argus* were collected by hand by using SCUBA from coral reefs near the Keys Marine Laboratory on Long Key, FL (24°49.567N, 80°48.884W) from April to August 2006. Lobsters were maintained in individual glass aquaria containing about 75 l of continuously aerated seawater (salinity of 35–36; 27°C) that was filtered to remove particles >5 μm. The water in the aquaria was changed daily at random times, and the lobsters were fed frozen squid and shrimp every other day. A 14:10-hr photoperiod that simulated the ambient light/dark cycle at the time of collection was employed.

Before experimentation, ovigerous females were classified according to the developmental stage of their egg masses. A small group of embryos (N=20) was removed from the egg mass and examined under a dissecting microscope. Only lobsters with embryos that would hatch within 24–36 hr were tested, as female responsiveness to chemical cues that induce larval release behavior increases with embryo maturity. Females with late-stage embryos are more responsive than females with early-stage embryos (Ziegler and Forward 2007).

Peptide Solutions All test chemicals were obtained from Sigma Chemical Company. The peptides used were chosen based on previous studies with brachyuran crabs (e.g., Forward et al. 1987; Rittschof et al. 1989; De Vries and Forward 1991a). These include dipeptides composed of various combinations of neutral, basic, and acidic amino acids (Table 1), as well as several tripeptides and two oligopeptides. According to Rittschof et al. (1985) and De Vries et al. (1991), bioassays that used solitary amino acids alone did not induce pumping in previous studies on brachyuran crabs. Therefore, we did not test individual amino acids.

Test solutions containing peptides were prepared immediately before use in aged offshore seawater (salinity 35), which had been filtered to remove particles >0.5 μm. Previous studies found that aged offshore water lacked chemical cues that induce larval release behaviors (e.g., Rittschof et al. 1985, 1989). A stock solution for each peptide was made at 10^{-4} M and diluted for testing. An aliquot of the peptide solution was mixed into 15 l of filtered offshore water to form the appropriate test concentration.

Pumping Assay The frequency of pleopod pumping upon exposure to the test compounds was used as a bioassay to quantify larval release behavior of the female. The general

Table 1 Pleopod pumping response measurements of ovigerous *P. argus* to dipeptides

Dipeptide	Response Threshold Concentration (M)	Maximum Response (%)	Effective Concentration Range (log units)
Neutral–basic			
Glycine–arginine (Gly–Arg)	10^{-9}	51.8	4
Alanine–arginine (Ala–Arg)	10^{-8}	39.7	3
Basic–basic			
Lysine–arginine (Lys–Arg)	10^{-6}	24.3	2
Histidine–lysine (His–Lys)	No response	–	–
Acid–basic			
Glutamic acid–lysine (Glu–Lys)	No response	–	–
Basic–neutral			
Lysine–glycine (Lys–Gly)	No response	–	–
Basic–acid			
Arginine–glutamic acid (Arg–Glu)	No response	–	–
Neutral–neutral			
Glycine–serine (Gly–Ser)	No response	–	–
Neutral–acid			
Glutamic acid–aspartic acid (Glu–Asp)	No response	–	–
Acid–neutral			
Glutamic acid–glycine (Glu–Gly)	No response	–	–
Acid–acid			
Glutamic acid–glutamic acid (Glu–Glu)	No response	–	–

experimental procedure was, first, to place an ovigerous female in a glass aquarium (50.8× 25.4 cm) containing 15 l of filtered (>0.5 μm) offshore seawater that did not contain the test solution. After a 5-min acclimation period, the number of pleopod movements was counted during a 1-min interval. This count is a measure of the *unstimulated* pumping rate. Second, the same lobster was transferred immediately to an identical aquarium with a test solution, and, after 5 min of acclimation, the number of movements in a 1-min period was counted. This second count was the *stimulated* pumping rate. An increase in pleopod pumping rate upon exposure to the test solution was considered a positive response. If pumping activity decreased or stayed the same, the lobster was scored as unresponsive.

For each test solution, a lobster was tested in a series of five or more concentrations, ranging from 10^{-4} to 10^{-12} M. The first solution in the series contained filtered offshore water (concentration=0 M), which served as a control (the unstimulated pumping rate). Lobsters were tested sequentially with the remaining concentrations proceeding from the lowest to highest to minimize the chances of sensory adaptation. Individuals were tested once at each test concentration. Each lobster was placed in filtered seawater (control) for at least 30 min before being retested at the next higher concentration. Preliminary experiments indicated that 30 min was sufficient time between exposures to test solutions for the lobster to return to the same pumping rate as in offshore water (i.e., unstimulated pumping rate). To ensure that test solutions remained active, solutions were remade after eight to ten lobsters were tested.

Pleopod pumping responses to the test peptides were characterized three ways: (1) response threshold concentration, (2) effective concentration range, and (3) maximum percent response (Rittschof et al. 1989). The response threshold concentration is defined as

the lowest concentration of a test substance to evoke a significant increase in pumping rate ($P<0.05$) relative to the pumping rate observed in the control (offshore water). The effective concentration range is the molar concentration range between the response threshold concentrations and the next higher concentration at which the pumping rate returns to the control level. The maximum percentage of response is the highest percentage of lobsters that responded to a test concentration within the effective concentration range (Forward et al. 1987; Rittschof et al. 1989).

The sample sizes for each test solution ranged from 12 to 20. Responses of lobsters to test solutions were compared to control responses (in filtered offshore water) by using a χ^2 test for multiple proportions and a Dunnet-type multiple comparisons test (Zar 1999).

Results

Pleopod Pumping Responses to Dipeptides Pleopod pumping responses by ovigerous *P. argus* to the test dipeptides varied with the amino acid composition of the peptide (Fig. 1). Pumping responses were elicited by all dipeptides with arginine (Arg) at the carboxy terminus (Table 1). Response threshold concentrations ranged from 10^{-6} M for Lys–Arg to 10^{-9} M for Gly–Arg. The maximum percent response ranged from 24.3 to 51.8%, with the greatest number of animals responding to Gly–Arg (Table 1). The effective concentration range for each active dipeptide was at least 2 log units of concentration, and was as high as 4 log units for Gly–Arg. Only dipeptides with a neutral–basic or basic–basic structure were active. No significant pumping responses were observed for any of the other dipeptide combinations tested (Table 1).

The dose-response curves for dipeptides that induced a significant response show a similar trend (Fig. 1). As concentration increases, pumping activity rises from a threshold to a maximum level, then declines at higher concentrations, sometimes to a non-significant level (e.g., Ala–Arg).

Pleopod Pumping Responses to Tripeptides Most of the test tripeptides with Arg at the carboxy terminus produced a significant pleopod pumping response, with the exception of

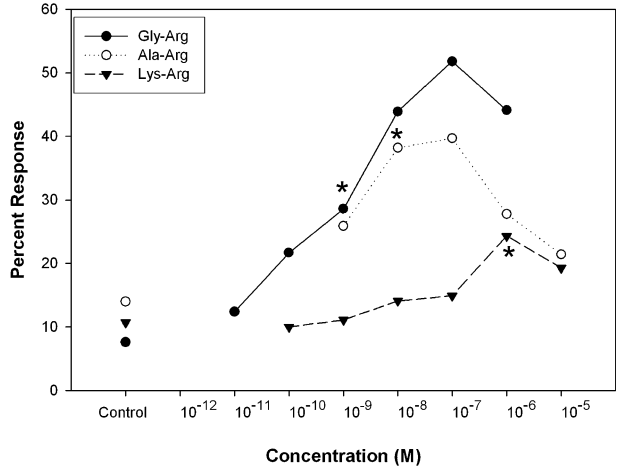


Fig. 1 Percentage of ovigerous *P. argus* responding to dipeptides. Control is the pumping response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly greater than the controls ($P<0.05$) is indicated by an *asterisk*. ($N=18$ for all test solutions)

Trp–Ile–Arg (Fig. 2). Response thresholds ranged from 10^{-8} M for Gly–Gly–Arg to 10^{-6} M for Pro–Ile–Arg (Table 2). The maximum response ranged from 38.4 to 58.3%, with the highest pumping response observed for Gly–Gly–Arg (Table 2). The effective concentration range for each tripeptides was at least 3 log units of concentration.

The neutral–basic-basic tripeptide Gly–His–Lys also produced a significant pumping response (Fig. 2), with a response threshold of 10^{-4} M and a maximum response of 26.1%, which was the lowest of all tripeptides tested (Table 2). There was no significant pumping response when lobsters were subjected to increasing concentrations of the neutral–neutral–basic tripeptide Gly–Gly–His (Fig. 2; Table 2). These results indicate that neutral-basic amino acid combinations of tripeptides with Arg or Lys at the carboxy terminus are capable of inducing a significant pleopod pumping responses for *P. argus* (Table 2).

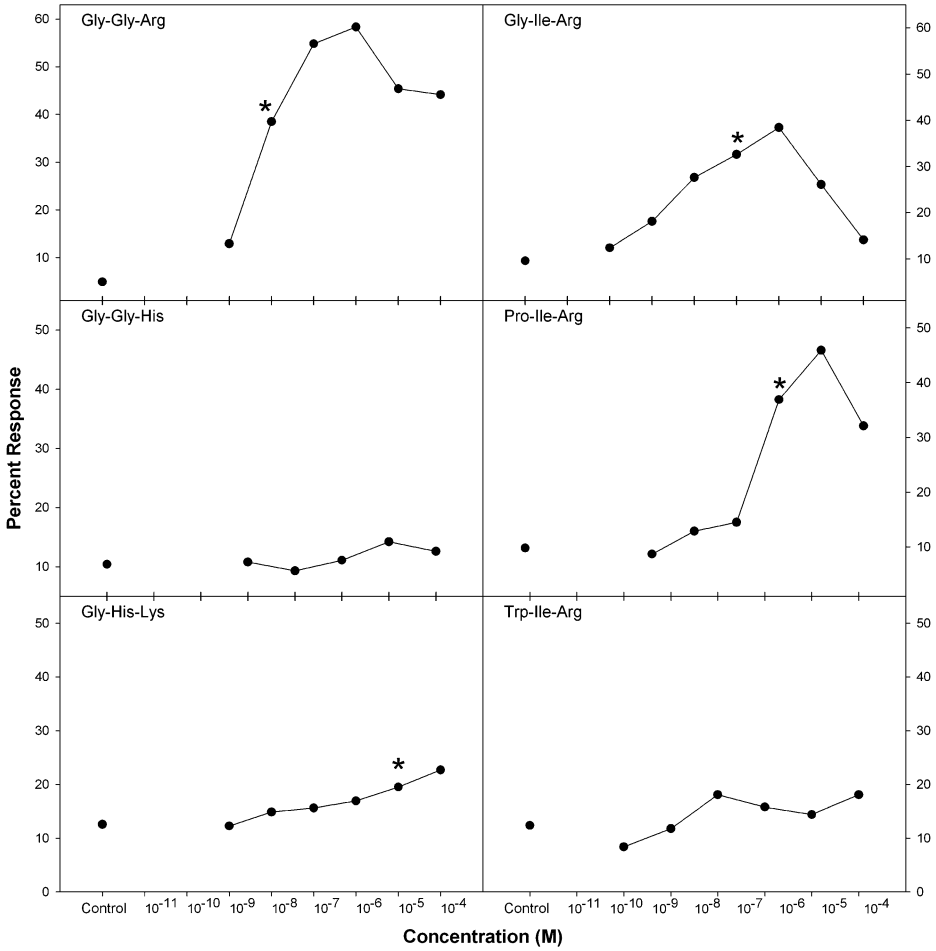


Fig. 2 Percentage of ovigerous *P. argus* responding to tripeptides. Control is the response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly greater than the controls ($P<0.05$) is indicated by an *asterisk*. ($N=12$ for Gly–Gly–His and Trp–Ile–Arg, $N=14$ for all other test solutions)

Table 2 Pleopod pumping response measurements of ovigerous *P. argus* to tripeptides

Tripeptide	Response Threshold Concentration (M)	Maximum Response (%)	Effective Concentration Range (log units)
Neutral–neutral–basic			
Glycine–glycine–arginine (Gly–Gly–Arg)	10^{-8}	58.3	4
Glycine–isoleucine–arginine (Gly–Ile–Arg)	10^{-7}	38.4	3
Proline–isoleucine–arginine (Pro–Ile–Arg)	10^{-6}	45.9	3
Tryptophan–isoleucine–arginine (Trp–Ile–Arg)	No response	–	–
Glycine–glycine–histidine (Gly–Gly–His)	No response	–	–
Neutral–basic–basic			
Glycine–histidine–lysine (Gly–His–Lys)	10^{-4}	26.1	1

The dose-response curves for tripeptides that induced a significant response show a similar trend (Fig. 2). As concentration increases, pumping activity rises from a threshold to a maximum level, then declines at higher concentrations, but rarely to a non-significant level. An exception is Gly–His–Lys, in which responses modestly increased with increasing concentration.

Pleopod Pumping Responses to Bradykinin Pleopod pumping rate increased when ovigerous lobsters were subjected to increasing concentrations of bradykinin, an oligopeptide with Arg at the basic carboxy terminus (Fig. 3). Pumping rates were not significantly different from the control when lobsters were subjected to increasing concentrations of des–Arg–bradykinin, an oligopeptide without a basic amino acid at the carboxy terminus (Fig. 3). The response threshold for bradykinin was 10^{-7} M, with a maximum response of 52.9% (Table 3). The effective concentration range for bradykinin was over 3 log units of concentration (Fig. 3; Table 3). These data suggest that the

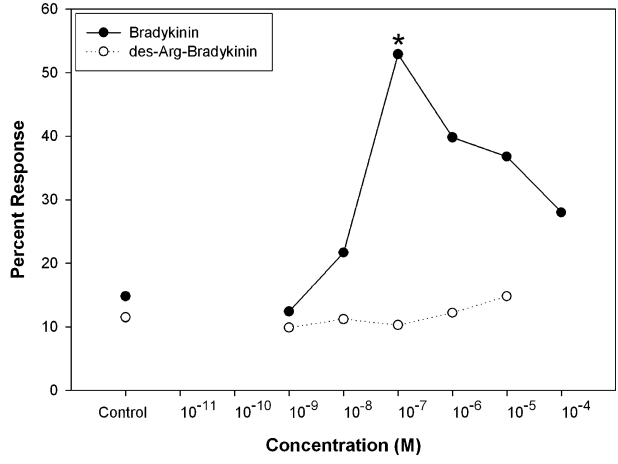


Fig. 3 Percentage of ovigerous *P. argus* responding to bradykinin (arginine at carboxy terminus) or des–Arg–bradykinin (lacking arginine at the carboxy terminus). Control is the response level upon exposure to filtered offshore water. Lowest concentration at which the pumping response was significantly greater than the controls ($P<0.05$) is indicated by an *asterisk*. ($N=20$ for both concentrations)

Table 3 Response measurements of ovigerous spiny lobsters to oligopeptide sequences with a basic carboxyl terminal (bradykinin) or without (Des–Arg^9–bradykinin)

Oligopeptide	Response Threshold Concentration (M)	Maximum Response (%)	Effective Concentration Range (log units)
Bradykinin Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	10^{-7}	52.9	3
Des–Arg^9–Bradykinin Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe	No response	–	–

oligopeptide bradykinin has the correct chemical format of neutral–basic amino acid combination at the carboxyl terminal, which is capable of inducing a significant pleopod pumping responses for *P. argus* (Table 3).

As the concentration of bradykinin increases, pumping activity rises from a threshold (10^{-7} M) to a maximum level, then declines (Fig. 3). This response pattern was similar to the curves for Gly–Arg, Gly–Gly–Arg, and Pro–Ile–Arg (Figs. 1, 2,and 3).

Discussion

Our model for larval release by the spiny lobster *P. argus* proposes that egg hatching readiness is communicated to the female by the release of peptide pheromones from the embryos (Forward and Lohmann 1983; Rittschof et al. 1985; Ziegler and Forward 2007). These pheromones induce the female to undergo stereotypical behaviors involving rapid movements of pleopods. This physical "pumping" activity assists in the rupture of the egg membranes and the subsequent release of free swimming larvae. Peptides that elicit a positive response are considered to be "active peptides". Our results show that specific peptides were capable of inducing the larval release behavior.

Peptides with Arg or Lys at the carboxy terminus and an adjacent neutral or basic amino acid were capable of inducing a pumping response in ovigerous *P. argus* (Tables 1, 2, and 3). The compounds with the lowest threshold concentration for inducing the pumping response were Gly–Arg (threshold at 10^{-9} M), followed by Gly–Gly–Arg and Ala–Arg (thresholds both at 10^{-8} M). Bradykinin was able to induce pleopod pumping in *P. argus* at a response threshold of 10^{-7} M. This result suggests that increased potency might be achieved by decreasing the length of the peptide. The shortest peptide (Gly–Arg) had the lowest threshold concentration.

In terms of response threshold, peptides with Arg at the carboxy terminus are more active than those containing Lys (Tables 1 and 2). Arg has an additional amino group, and thus, is more basic than Lys. In addition, Lys has a shorter chain length (R group) with a lower pK_a. Such structural differences may explain the differences in response levels. The basic tripeptide Gly–His–Lys produced a significant pumping response (Fig. 3); however, the concentration necessary to produce a significant response was $>10^{-4}$ M. This is quite high in comparison to peptides with Arg. Active peptides containing Lys at the carboxy terminus were able to induce a significant response in the mud crab *R. harrisii*, but only at threshold levels that were higher than amounts possible in the natural pumping pheromones (Rittschof et al. 1985; Forward et al. 1987). Thus, peptides containing Lys at the carboxy terminus may not be used as pheromones for *P. argus*.

The peptides tested that induced significant responses were at similar concentrations to the concentrations predicted for the native pheromones in brachyuran crabs (Forward et al. 1987; Rittschof et al. 1989). The lower threshold for the most active peptides tested (Gly–Arg, Gly–Gly–Arg, Ala–Arg, and bradykinin) ranged from 10^{-7} to 10^{-9} M for *P. argus*. These thresholds are 3–4 orders of magnitude below those peptides used as pheromones by other marine invertebrates such as the sand dollar *Dendraster excentricus* (Burke 1984), the abalone *Haliotis* (Morse 1988), the barnacle *Balanus amphitrite* (now known as *Amphibalanus amphitrite*; Tegtmeyer and Rittschof 1989), and the cnidarian *Cassiopeia andromeda* (Fleck 1998; Walther and Fleck 1998). Thus, sufficient amounts of these peptides could be released by the eggs to serve as pheromones.

The chemical induction of the behavioral response occurs only within a narrowly defined concentration range. Responses typically decline when the concentrations are either more or less that one magnitude from the concentration that evokes the greatest responses (Forward et al. 1987; Rittschof et al. 1989; Browne et al. 1998; Decho et al. 1998). Peptides such as Ala–Arg, Gly–Ile–Arg, and bradykinin are inhibitory at high concentrations. This inhibition may be caused by inhibition at the neural level (Derby and Atema 1988; Rittschof et al. 1989).

These results are consistent with previous studies of chemically induced larval release in crustaceans. The chemical nature of pumping pheromones for brachyuran crabs has been determined to be a heterogeneous group of small peptides containing Arg (Rittschof et al. 1985, 1989; Forward et al. 1987). Behaviorally active peptides have a general structure of a neutral amino acid at the amino terminus (e.g., alanine) coupled to a basic amino acid at the carboxy terminus (e.g., arginine; Forward et al. 1987; Rittschof et al. 1989; Pettis et al. 1993). Other common characteristics of these active peptides include a positively charged R group attached to the carboxy-terminal and a hydrophobic R group attached to the amino-terminal end of the molecule (Pettis *et al.* 1993).

Peptides capable of inducing the pumping response in the crab *R. harrisii* have Arg as the carboxy terminal residue and a neutral amino acid at the hydrophobic residue (Forward et al. 1987). An amino acid analysis of the pumping factors previously indicated that peptides with histidine (His) were not among those associated with *R. harrissi* (Rittschof et al. 1985), although Gly–His–Lys produced a significant pumping response (Forward et al. 1987). This is consistent with our results for *P. argus* in which the tripeptide Gly–His–Lys produced a significant pumping response, whereas Gly–Gly–His did not. These results suggest that His cannot be the carboxy terminal residue in the peptide pheromone.

Although His is a basic amino acid, it contains an imidazole ring structure, and thus, may lack the necessary components to act as a chemical signal in this system. Lys and Arg are more basic amino acids than His. The pK_a values for Lys and Arg indicate that their side chains are always positively charged under physiological conditions. These amino acids are strongly polar, and, as a consequence, can easily be hydrated by the surrounding aqueous environment. Histidine has a pK_a in physiological range and is often incorporated into protein active sites. The imidazole can exchange protons at physiological (neutral) pH, and thus, it often plays a role in enzymatic catalysis (Mathews et al. 1999).

Peptides with Arg or Lys at the carboxyl terminus are produced by trypsin-like serine proteases, which cleave after basic amino acids. Therefore, the pheromones that induce pumping behavior may be generated by trypsin-like serine proteases capable of digesting the membrane around the embryos of spiny lobsters. Trypsin-like activity may result from enzymes released by embryos (Rittschof et al. 1990a, b; De Vries and Forward 1991b). The brachyuran crabs *Neopanopae sayi*, *Uca pugilator*, and *Sesarma cinereum* release proteolytic enzymes near the time of hatching (De Vries and Forward 1991b). These assist

with the breakage of the outer membrane of the egg case, resulting in the emergence of a free-swimming larva. A logical extension of our work is to test the hypothesis that peptide pumping pheromones are created by enzymatic activity (Rittschof et al. 1990a, b).

Results from the present study support the hypothesis that larval release behaviors are induced by active substances (pheromones) composed of small peptides. The active peptides likely have a specific amino acid sequence of neutral–basic and neutral–neutral–basic di-, tri- and oligopeptides. These biologically potent peptides are effective at concentrations from 10^{-4} to 10^{-9}. The results of this study support the conceptual model that larval release in spiny lobster *P. argus* is controlled by pheromones released from hatching eggs that act to induce the stereotypical pumping behaviors of the female, synchronizing larval release.

Releasing larvae in synchrony with the time of slack water before the ebbing tide allows the phyllosoma larvae to enter the water column at a beneficial time for transport by currents to offshore nursery areas for development (Yeung and Lee 2002). This synchronous timing also ensures that larvae are released at times that minimize the threat of visual predation and exposure of larvae to adverse conditions such as low salinity and high temperatures (Forward 1987).

Acknowledgements This material is based in part on research supported by the National Science Foundation grant number OCE-0221099 and ECOHAB grant number NA170P2725, with additional funding from the Oak Foundation. All research was conducted according to the conditions of the FFWCC Special Activities License #05SR-9340. We thank Lonny Anderson, Dr. Mary Alice Coffroth, Todd Hitchins, Matthew Hoxie, Karen Neely, Daniel Poland, and Adrianna Zito for support with lobster collection. Gary Dickinson and Joshua Osterberg provided valuable comments to the manuscript. We also thank Dr. Dan Rittschof for helpful suggestions with the design and analyses of these experiments.

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Skin (epidermal) glands, the blood–skin barrier, cloacal glands, feces, and urine have been described as potential sources of pheromones in lizards (Mason 1992). In these animals, epidermal glands, and in particular, the femoral glands (holocrine, tubulo-follicular, sebaceous-type glands) in the femoral and pre-cloacal regions, have received the most attention (Quay 1986; Van Wyk and Mouton 1992; Dujsebayeva 1998). Another type of epidermal gland, the "generation" gland [mostly holocrine secretory cells located in the beta-layer of the epidermis (Maderson 1967; Van Wyk and Mouton 1992)], is also known to occur in the femoral, pre-cloacal, antebrachial (fore-arm), and dorsal epidermal regions in cordylid lizards (Van Wyk and Mouton 1992).

Reports regarding the chemical nature of the material secreted by the epidermal glands of lizards are limited to studies on the femoral and pre-cloacal glands (Alberts 1990; Weldon et al. 1990; Alberts et al. 1992, 1993; Escobar et al. 2001, 2003; López and Martín 2005a, b, 2006). Alberts (1990) reported proteins and lipids as the main components of the femoral secretion of the desert iguana, *Dipsosaurus dorsalis*, in a ratio of 4:1, respectively. The composition of the proteins varied among individuals. These proteins absorb ultraviolet light and may be used to facilitate visual detection of the deposited secretion by conspecifics (Alberts 1990; Alberts et al. 1993). In addition, volatile lipids could also act as olfactory cues for the detection of femoral secretions (Alberts 1990). In male green iguanids, Alberts et al. (1992) found seasonal variations in the lipid composition, but in contrast to the proteins, little individual variation was observed. Volatile lipids in these secretions could have a semiochemical function (Mason and Gutzke 1990; Escobar et al. 2001, 2003).

Alberts et al. (1992) identified a number of lipids, including carboxylic acids and steroid alcohols, in the femoral secretion of the lizard, *Iguana iguana*. Escobar et al. (2001, 2003) reported 49 lipids in 20 *Liolaemus* species. As in other lizard species (Chauhan 1987; Alberts 1990; Alberts et al. 1992), these compounds belong to three main classes, alkanes, carboxylic acids, and steroids. Information on a possible pheromonal role of the epidermal gland secretions of squamates is limited. Variation in the incidence and relative amounts of specific compounds has, nevertheless, been attributed to intraspecific (Alberts 1990; Escobar et al. 2003), interpopulational (Escobar et al. 2003), and interspecific differences (Cooper and Vitt 1986; Escobar et al. 2001).

If one accepts that chemicals in the femoral secretions play a role in the communication of lizards, then this raises the question of whether the major constituents, such as the fatty acids that are almost ubiquitous in nature, are the major semiochemicals of these animals, or whether minor components, perhaps overlooked in the investigations, are involved? To shed more light on this question, we started analyzing lizard secretions by using highly sensitive chemical methods.

The family Cordylidae (girdled lizards) is one of the lesser-known lizard families of the world and is restricted to the southern subcontinent of Africa (Frost et al. 2001). Femoral glands occur on the ventral aspect of the thigh in males, and in some species, also in the females (Mason 1992). The cordylid femoral glands correspond to the tubulo-follicular sebaceous type holocrine glands found in other lizards (Cole 1966; Quay 1986). The so-called sungazer, *Cordylus giganteus*, a large terrestrial girdled lizard, inhabits the Highveld *Themeda* grasslands in parts of the Free State, KwaZulu-Natal, and Mpumalanga provinces of South Africa (Van Wyk 1992; Branch 1998). Both sexes possess femoral glands in the ventral femoral region of the hind legs.

Males also exhibit generation glands as a patch of glandular scales, anterior to the single row of femoral glands. In addition, males have a patch of generation glands on the ventral aspect of the antebrachial (fore-arm) region (Van Wyk and Mouton 1992). The secretions of

these glands were not investigated in the present study. Due to the nature of their grassland habitat, visual communication may be of limited value, and therefore, chemical communication is a possible alternative. *C. giganteus* is also recognized as a species showing high refuge fidelity (Ruddock 2000) in a rather homogenous habitat, further suggesting the use of chemical cues for site recognition.

Here, we describe a comprehensive chemical characterization of the volatile constituents of the femoral gland secretions of *C. giganteus*. For the first time, the chemical properties of the secretions of a lizard species of the family Cordylidae are reported.

Methods and Materials

General

All Pyrex glassware was thoroughly cleaned with water and acetone, and heated to 500°C to remove any residual organic material. Dichloromethane (Fluka, Residue Analysis Grade) was used for extraction of organic material from collected samples. Syringes were cleaned with the same solvent.

Collection and Sample Preparation

Femoral gland secretions were collected from individual adult male and female lizards in a study population on a farm in the Lindley district (Free State Province, South Africa; 28°01′S, 28°05′E) during the months of April 1998 (11 males and 12 females), October 1998 (5 males and 5 females), and January 1999 (5 males and 5 females). Secretions were collected by squeezing plugs of the secretion from the femoral pores on the hind legs of the lizards, by using a pair of forceps. The collected material was transported to Stellenbosch at −10°C and stored at −70°C until processed for analysis.

Organic constituents were extracted with 150 μl of dichloromethane from secretions of individual lizards. The extracts were filtered and concentrated to ca 10 μl by slow evaporation in a nitrogen atmosphere. Samples of 0.2 to 1.0 and 1.0 μl, of the concentrated extracts were used for qualitative and quantitative analyses, respectively.

For the determination of the amount of each chemical in the femoral gland secretion, the secretion (11.44 or 10.89 mg, respectively) of a male or a female lizard was extracted with four portions of 200 μl dichloromethane. The extract was slowly evaporated to dryness in an inert atmosphere at 22°C, and the residual material dissolved in 20 μl of an internal standard solution containing 49 μg of octadecane/ml in dichloromethane. One mircoliter of each of these solutions was used for the analysis by GC.

Analytical Techniques

Gas chromatographic (GC) analyses were performed on Carlo Erba 4160 and 5300 GCs, while the quantitative analysis was carried out with an HP 5890 Series II GC. All instruments were equipped with a flame ionization detector (FID) and split/splitless inlet systems, with the injector and detector at 220 and 280°C, respectively. Helium was used as carrier gas at a linear flow velocity of 28.6 cm sec^{-1}. Glass capillary columns (40 m × 0.3 mm i.d.) were coated with PS-089-OH [silanol-terminated (95%)-methyl-(5%)-phenylpolysiloxane copolymer] at a film thickness of 0.25 μm. Samples were injected in the split mode by using a split ratio of 1:10. The volatile chemicals were thermally focused on the column at a temperature of 30°C, after

which, the column was heated ballistically to 40°C and then programmed at 8°C min^{-1} from 40 to 160°C, followed by 2°C min^{-1} to 280°C (hold 60 min).

Electron impact mass spectra (EI-MS) were acquired at 70 eV on a Carlo Erba QMD 1000 GC-MS instrument, using the same column and conditions as above. For the analysis of steroid derivatives, a mass range of *m/z* 25–550 was selected. An ion source temperature of 200°C and an interface temperature of 250°C were used for all analyses. Some GC-MS analyses were carried out on a Fisons MD-800 instrument using the parameters specified above.

High-resolution GC-MS analyses of an extract from a male sungazer were performed on an AMD Intectra 604 high-resolution mass spectrometer coupled to a Carlo Erba 4160 gas chromatograph using the gas chromatographic parameters specified for the QMD 1000 instrument.

Trimethylsilyl Derivatives

An extract of femoral gland secretion was concentrated in a Reacti-Vial left uncapped in a nitrogen atmosphere to yield approximately 1 mg of solvent-free material. Methoxylamine hydrochloride (5.23 mg, 0.0626 mmol) dissolved in pyridine (50 μl) was added to the material. The reaction mixture was heated in a GC oven for 15 min at 60°C, after which, the solvent was carefully blown off with nitrogen. Trimethylsilylimidazole (75 μl, 0.511 mmol) was added to the reaction product and the reaction mixture heated at 100°C for 2 hr in a capped vial (Thenot and Horning 1972). The sample was concentrated to about 50 μl in a nitrogen atmosphere while the reaction product was still hot. Approximately 2 μl of this solution was used for a GC-MS analysis.

Reference Compounds

Most of the compounds used to confirm the identity of the chemicals of the femoral gland secretions were obtained commercially or were available from previous studies. The following compounds were synthesized.

Dodecyl propenoate (**2**) was synthesized by a transesterification procedure described by Rehberg (1955). Dodecanol (16.3 g, 87.5 mmol) and methyl propenoate (25.0 g, 291 mmol) were refluxed in a nitrogen atmosphere in the presence of *p*-toluenesulfonic acid monohydrate (2.00 g, 10.5 mmol), as catalyst, and hydroquinone (0.350 g, 3.18 mmol) as radical scavenger. After refluxing the reaction mixture for 30 min, the temperature was reduced to 62–63°C, and the methanol/methyl propenoate azeotrope formed in the transesterification reaction distilled off over a period of 4.5 hr. The temperature was increased, and the residual methyl propenoate distilled off at 80°C, yielding dodecyl propenoate (20.654 g, 98% yield) in a purity of 97%. MS (70 eV): *m/z* (%) 168(2), 140(4), 127(9), 125(4), 113(9), 111(13), 97(23), 83(30), 73(32), 69(32), 55(100), 43(45), 41(52), 39(10), 29(28), 27(32).

Dodecyl propanoate (**3**) was likewise synthesized by the Rehberg method from dodecanol and methyl propanoate to give the target compound. MS (70 eV): *m/z* (%) 213 (1), 168(2), 140(4), 130(2), 125(3), 111(12), 97(28), 83(39), 75(73), 57(100), 55(43), 43 (36), 41(31), 39(4), 29(24), 27(5).

Results and Discussion

GC-MS analyses of the femoral gland secretions of male and female sungazers gave total ion chromatograms (TICs) that were qualitatively identical but with some

quantitative differences. A typical total ion chromatogram of an extract of the femoral gland secretion of a female sungazer is depicted in Fig. 1. The extracts of the secretions of both male and female lizards did not contain any early eluting volatile constituents. To rule out the possibility that highly volatile material present in the secretion could have been lost when the sample was concentrated for analysis, GC-MS analysis was repeated by using solventless sample introduction (Burger et al. 1990) of the raw secretion. Results confirmed that the secretion, as collected, was devoid of more volatile compounds commonly present in exocrine secretions and that the constituents of the secretion can be described as semi-volatile.

A total of 173 chromatographic peaks were observed in the GC-FID analyses, but in the GC-MS analyses, only 53 components (Table 1) could be detected with sufficient sensitivity to yield mass spectra suitable for identification. The quantitative GC-FID analyses showed that these 53 compounds constituted more than 90% of the total mass of the detected compounds. The components of the extract were tentatively identified by comparison of their low-resolution electron impact mass spectra with NBS, NIST, Wiley, and the in-house LECUS mass spectra libraries. Further diagnostic information on some tentatively identified steroids was obtained by interpretation of the mass spectra of their

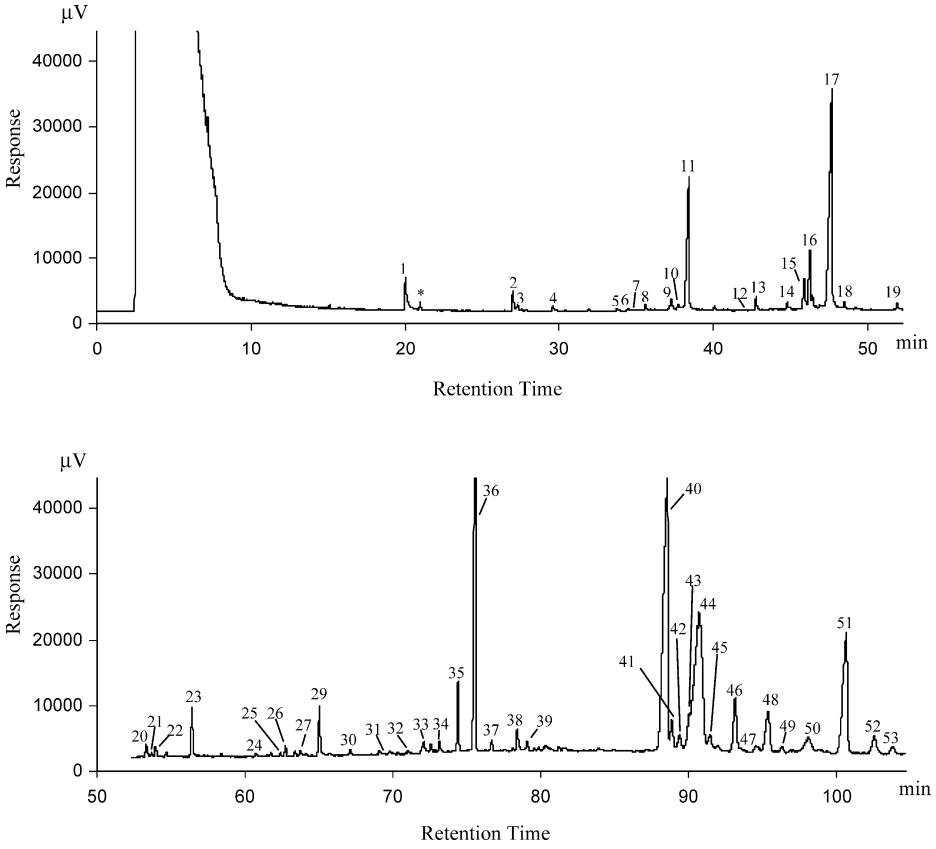


Fig. 1 Total ion chromatogram (FID) of an extract of femoral gland secretion of a female sungazer, *C. giganteus*. *Contaminant

Table 1 Constituents identified in the femoral gland secretion of the sungazer

Number in Fig. 1[a]	Constituent	Ident.[b]	Quantity (ng/mg)[c]		Male relative Amounts ± S[d]			Female relative Amounts ± S[d]		
			Male	Female	April 1998 (n=11)[e]	October 1998 (n=5)[e]	January 1999 (n=5)[e]	April 1998 (n=12)[e]	October 1998 (n=5)[e]	January 1999 (n=5)[e]
Acids										
4	Tetradecanoic acid	a,b	179	158	0.25±0.08	0.35±0.23	0.23±0.09	0.15±0.06	0.13±0.05	0.14±0.05
5	Pentadecanoic acid	a,b	135	125	0.12±0.03	0.15±0.06	0.11±0.06	0.12±0.04	0.16±0.02	0.18±0.13
9	9*Z*-Hexadecenoic acid	a,b	468	191	0.76±0.39	0.94±0.70	0.75±0.28	0.34±0.23	0.27±0.10	0.39±0.15
10	Unidentified (a hexadecenoic acid)	a,c,d	62	69	0.14±0.07	0.19±0.15	0.13±0.06	0.25±0.13	0.35±0.21	0.35±0.30
11	Hexadecanoic acid	a,b	4,209	2,318	7.05±2.05	8.59±3.03	7.16±2.68	4.82±1.44	4.76±1.15	4.42±2.02
12	Unidentified (a heptadecenoic acid)	a,c,d	32	21	0.03±0.03	0.03±0.01	0.02±0.02	0.05±0.06	0.02±0.01	0.02±0.01
13	Heptadecanoic acid	a,b	188	170	0.27±0.08	0.27±0.08	0.24±0.08	0.50±0.18	0.55±0.03	0.42±0.17
15	9*Z*,12*Z*-Octadecadienoic acid	a,b	394	282	1.31±0.62	1.22±0.59	1.54±0.63	1.26±0.68	1.48±0.47	1.71±0.45
16	9*Z*-Octadecenoic acid	a,b	1,321	1,042	1.31±0.45	1.81±0.42	1.88±0.80	2.24±1.11	2.57±0.63	3.31±1.12
17	Octadecanoic acid	a,b	2,989	3,313	6.80±1.43	6.62±1.91	6.44±2.36	10.74±3.48	12.37±3.60	10.06±5.23
19	Nonadecanoic acid	a,b	37	40	0.12±0.02	0.16±0.06	0.12±0.04	0.18±0.07	0.22±0.07	0.16±0.06
20	8*Z*,11*Z*,14*Z*-Icosatrienoic acid	a,c	488	270	2.27±1.58	1.49±1.08	2.64±0.79	0.69±0.46	0.29±0.11	1.07±0.39
23	Icosanoic acid	a,b	134	250	0.66±0.12	0.87±0.21	0.77±0.23	1.17±0.47	1.67±0.69	1.14±0.60
24	Henicosanoic acid	a,b	32	68	0.06±0.02	0.09±0.04	0.07±0.02	0.11±0.05	0.17±0.09	0.10±0.04
28	Unidentified (a docosenoic acid)	a,c,d	240	341	0.08±0.04	0.10±0.03	0.16±0.08	0.08±0.04	0.17±0.04	0.17±0.03
29	Docosanoic acid	a,b	38	110	0.64±0.15	0.91±0.34	0.84±0.24	1.20±0.55	1.83±0.79	1.18±0.68
31	Tricosanoic acid	a,b	44	74	0.01±0.01	0.01±0.01	0.00±0.00	0.02±0.02	0.02±0.02	0.02±0.02
33	Unidentified (a tetracosenoic acid)	a,c,d	96	85	0.13±0.04	0.20±0.19	0.20±0.13	0.17±0.11	0.18±0.13	0.20±0.13
34	Tetracosanoic acid	a,b	50	91	0.18±0.10	0.28±0.14	0.25±0.09	0.29±0.17	0.52±0.26	0.34±0.22
Alcohols										
1	1-Dodecanol	a,b	30	17	1.37±0.84	0.01±0.01	0.02±0.02	2.43±1.18	–	0.17±0.26
7	1-Hexadecanol	a,b	18	25	0.03±0.01	0.05±0.02	0.04±0.01	0.03±0.01	0.04±0.01	0.03±0.01
Ketones										
8	2-Heptadecanone	a,b	210	230	0.13±0.05	0.11±0.06	0.10±0.02	0.28±0.12	0.33±0.06	0.28±0.08
14	2-Nonadecanone	a,b	312	213	0.19±0.07	0.17±0.06	0.17±0.06	0.27±0.12	0.36±0.08	0.26±0.08

Table 1 (continued)

Number in Fig. 1[a]	Constituent	Ident.[b]	Quantity (ng/mg)[c]		Male relative Amounts ± S[d]			Female relative Amounts ± S[d]		
			Male	Female	April 1998 (n=11)[e]	October 1998 (n=5)[e]	January 1999 (n=5)[e]	April 1998 (n=12)[e]	October 1998 (n=5)[e]	January 1999 (n=5)[e]
22	2-Henicosanone	a,b	43	42	0.22±0.06	0.22±0.06	0.23±0.06	0.44±0.16	0.49±0.10	0.39±0.08
26	2-Tricosanone	a,b	255	202	0.21±0.06	0.22±0.07	0.21±0.06	0.53±0.15	0.51±0.13	0.41±0.12
30	2-Tetracosanone	a,b	51	57	0.02±0.02	0.06±0.03	0.02±0.02	0.14±0.09	0.61±0.31	0.21±0.12
32	2-Pentacosanone	a,b	83	72	0.06±0.02	0.05±0.03	0.05±0.02	0.13±0.06	0.14±0.06	0.09±0.03
Esters										
2	Dodecyl propenoate	a,b	357	560	0.43±0.15	–	0.06±0.02	0.80±0.35	–	0.26±0.30
3	Dodecyl propanoate	a,b,g	33	63	0.09±0.03	–	0.01±0.00	0.17±0.07	0.00±0.01	0.02±0.02
Steroids										
27	Unident. ster. (217, 232, 246, 259, 302)[f]	a	124	123	0.01±0.01	0.06±0.04	0.02±0.02	0.13±0.07	0.32±0.18	0.26±0.18
37	Unident. ster. (135, 143, 247, 265, 366)	a	465	206	0.39±0.17	1.12±0.80	0.75±0.51	0.33±0.14	0.60±0.22	0.57±0.18
38	Unident. ster. (155, 197, 251, 349, 364)[g]	a	371	506	0.44±0.27	0.55±0.25	0.32±0.14	0.93±0.18	1.17±0.31	0.89±0.43
39	Unident. ster. (195, 209, 249, 349, 364)	a	140	132	0.07±0.05	0.08±0.02	0.07±0.02	0.29±0.07	0.33±0.07	0.26±0.09
40	Cholest-5-en-3β-ol	a,b,e	7,279	7,155	10.44±2.44	15.94±3.19	10.10±2.37	15.46±3.43	18.74±5.63	15.57±6.97
41	Unident. ster. (217, 233, 351, 384, 388, 441, 456)	a	626	656	0.47±0.10	0.63±0.11	0.56±0.11	0.96±0.36	0.98±0.34	1.21±0.38
42	Unident. ster. (213, 229, 273, 353, 371, 386)	a	1,709	217	0.07±0.03	0.10±0.07	0.07±0.02	0.34±0.15	0.75±0.27	0.22±0.08
44	Cholesta-5,7-dien-3β-ol	a,b,e	6,783	3,703	5.44±2.07	4.06±1.76	4.78±2.64	16.07±4.64	11.54±5.49	15.11±4.64
45	Unident. ster. (213, 237, 335, 350, 368)	a	69	144	0.13±0.08	0.10±0.08	0.11±0.06	0.40±0.16	0.45±0.15	0.28±0.20
46	Unident. ster. (259, 273, 340, 381, 399, 414)	a,e	2,066	1,080	2.22±0.87	3.45±1.39	2.59±0.87	1.84±0.86	2.20±0.26	2.56±0.98

47	Unident. ster. (231, 243, 257, 275, 397, 412)	a	137	295	0.27±0.06	0.45±0.24	0.34±0.13	0.38±0.18	1.09±0.92	0.56±0.40
48	Ergost-5-en-3β-ol	a,e	1,048	946	1.18±0.31	1.96±0.48	1.40±0.25	1.96±0.49	2.35±0.46	2.10±0.97
49	Cholest-4-en-3-one	a	110	666	0.07±0.06	0.26±0.20	0.20±0.33	0.23±0.32	2.82±4.48	1.45±3.00
50	Unident. ster. (257, 271, 339, 365, 398, 411, 426)	a	901	878	1.04±0.29	1.24±0.58	1.03±0.44	1.49±0.54	3.38±2.57	1.84±0.74
51	Lanost-8-en-3β-ol	a,e	7,761	4,212	15.13±4.20	18.32±4.30	14.57±2.55	8.60±3.28	7.68±1.98	9.00±3.29
52	Stigmast-5-en-3β-ol	a,e	368	236	0.51±0.24	0.77±0.26	0.59±0.17	0.79±0.35	1.27±0.38	1.00±0.35
53	Lanosterol	a,b,e	532	360	0.72±0.15	1.46±0.77	0.94±0.16	0.48±0.28	0.62±0.25	0.61±0.20
Other										
6	2-Heptadecenal	a,c,f	209	67	0.11±0.06	0.21±0.12	0.13±0.03	0.07±0.04	0.11±0.03	0.06±0.04
21	Unidentified alkene	a	28	15	0.05±0.05	0.06±0.07	0.06±0.03	0.08±0.06	0.02±0.01	0.04±0.03
25	Pentacosane	a,b	62	63	0.01±0.01	0.02±0.02	0.02±0.01	0.13±0.09	0.03±0.03	0.05±0.02
36	Squalene	a,b	1,5359	2,376	30.50±4.86	17.76±8.10	31.42±5.58	8.57±2.28	3.67±1.22	9.33±2.85
43	α-Tocopherol	a,b	606	323	1.71±0.83	0.99±0.53	1.82±0.99	2.14±1.54	1.61±1.00	2.64±1.21
18	Unidentified (185, 222, 264, 284, 296)	a	131	169	0.01±0.01	0.02±0.01	0.01±0.01	0.06±0.04	0.06±0.04	0.07±0.04
35	Unidentified (111, 125, 153, 243, 253, 282)	a	80	155	0.23±0.20	0.34±0.14	0.16±0.07	1.15±1.62	0.62±0.40	0.65±0.55

a GC-MS analysis, *b* retention time comparison with synthetic compounds, *c* retention time increment comparison, *d* position of double bond unknown, *e* EI-spectra of TMS derivatives, *f* *E*/*Z*-isomerism unknown, *g* GC-HRMS

[a] In order of elution from GC column

[b] Identification

[c] Mass of each chemical in 1 mg of secretion

[d] Relative amounts are calculated as a percentage of the total 173 compounds detected by GC-FID. The mean ± SD is reported.

[e] The same five lizards were sampled in each of the three seasons or were present in the larger samples

[f] Prominent ions in the mass spectra of unidentified steroids

[g] Ions corresponding to those in the mass spectrum of an unidentified steroid found in the femoral gland secretion of *Podarcis hispanica* (Martín and López 2006b).

trimethylsilyl derivatives, employing published mass spectral data. The structures of most of these compounds were confirmed by gas chromatographic co-elution with authentic reference compounds.

Although a large number of homologous long-chain alkanes were identified in the precloacal secretions of 20 *Liolaemus* lizard species (Escobar et al. 2001, 2003), alkanes have not been found in the secretions of other lizards. The femoral secretion of *C. giganteus* contained only one alkane, pentacosane.

Constituents **1** and **7** were tentatively identified as long-chain alcohols. The molecular mass of long-chain alcohols can normally be established by invoking a characteristic ion at $[M-46]^+$ formed by the loss of a molecule each of water and ethylene. Although constituent **7** was present in such a low concentration that this ion could not be detected, this chemical and constituent **1** were identified by gas chromatographic co-elution with authentic synthetic reference compounds as 1-hexadecanol and 1-dodecanol, respectively. These were the only alcohols identified in the secretions. The femoral secretions of some other species that have been investigated were found to contain up to nine homologous unbranched long-chain alcohols (López and Martín 2005b; Martín and López 2006b).

A series of methyl ketones, comprising 2-heptadecanone (**8**), 2-nonadecanone (**14**), 2-henicosanone (**22**), 2-tricosanone (**26**), 2-tetracosanone (**30**), and 2-pentacosanone (**32**), was identified in the femoral secretion of the sungazer. Previous studies have found C_{13} and C_{19}, C_{15} and C_{16}, C_{16} and C_{19}, and C_{19} ketones in the femoral secretions of, respectively, *Acanthodactylus erythrurus* (López and Martín 2005b), *Lacerta schreiberi* (López and Martín 2006), *Psammodromus algirus* (Martín and López 2006a), and *Podarcis hispanica* (Martín and López 2006b),

The sungazer's femoral secretion contained a single unsaturated C_{17} aldehyde. Martín and López (2006a, b) found only a few aldehydes in the secretions of the species they studied. Aldehydes are oxidized by oxygen to fatty acids in an autoxidation reaction that is promoted by high temperatures and ultraviolet light (Pryor 1966). It is possible that α-tocopherol, a radical scavenger (Fossey et al. 1995) present in the femoral secretion of lizards (e.g., *Lacerta schreiberi*; López and Martín 2006), largely inhibits this reaction.

The secretions contained 19 saturated and unsaturated C_{14}–C_{24} carboxylic acids. Ions characteristic of carboxylic acids are present in low abundance in the mass spectra of constituents **10**, **12**, **28**, and **33**. Based on the available mass spectral information and retention time comparison with other saturated and unsaturated carboxylic acids, these compounds were identified as unsaturated carboxylic acids. However, the position of unsaturation in these acids was not established. Previous investigators (e.g., Alberts et al. 1992) have found carboxylic acids with similar chain lengths in other species, while still other species produce carboxylic acids with a wider range in the number of carbon atoms (e.g., C_6–C_{26} in *Liolaemus* spp; Escobar et al. 2001).

The mass spectrum of constituent **3** contained ions which are typically present in the mass spectra of long-chain alcohols and alkenes. However, comparison of this spectrum with mass spectral libraries provided no conclusive characterization: the main point of divergence being the presence (in **3**) of an abundant ion at *m/z* 75. High-resolution GC-MS analysis revealed that the elemental compositions of this ion and another at *m/z* 57 were $C_3H_7O_2$ and C_3H_5O, respectively, indicating that constituent **3** could be an ester of propanoic acid, possibly dodecyl propanoate. The high-resolution mass spectrum of constituent **2** showed that the prominent ions at *m/z* 73 and 55 were $C_3H_5O_2$ and C_3H_3O, respectively. As polar compounds elute faster than analogous saturated compounds on apolar columns, it was concluded that constituent **2** is the propenoic acid ester analog of constituent **3**. Gas chromatographic co-elution of these two chemicals with synthetic

Table 2 Diagnostic ions and normalized abundances[a] from the mass spectra of the TMS derivatives of the steroids identified in the femoral gland secretion of the sungazer

TMS-derivative of:	M	M-15[b]	M-90[c]	M-(90+15)	M-129[d]	M-(sc.[e] + 90)	M-(D-ring[f] + 90)	M-(C-ring[g] + 90)
Cholest-5-en-3β-ol	458	443	368	353	329	255	213	145
	7[a]	*2*	*26*	*15*	*32*	*14*	*10*	*33*
Cholesta-5,7-dien-3β-ol	456	441	366	351	a	253	211	143
	5	*≥0*	*16*	*70*	–	*18*	*25*	*64*
Ergost-5-en-3β-ol	472	457	382	367	343	255	213	145
	4	*2*	*24*	*10*	*24*	*9*	*9*	*28*
Lanost-8-en-3β-ol	500	485	410	395	–	297	255	173/175
	5	*10*	*2*	*100*	–	*2*	*9*	*20/19*
Stigmast-5-en-3β-ol	486	471	396	381	357	255	213	145
	5	*2*	*17*	*8*	*18*	*11*	*10*	*34*
Lanosterol	498	483	(408)[h]	393	–	297	255	173/175
	5	*9*	–	*38*	–	*3*	*14*	*13/9*

a M-131 is however formed at *m/z* 325, with a relative abundance of 62%

[a] Normalized abundances are given in italics

[b] Loss of a methyl group

[c] Loss of TMSOH

[d] Loss of $TMSO(CH)_2CH_2$

[e] sc. = side chain on C17

[f] Cleavage of the D-ring

[g] Cleavage of the C-ring

[h] Does not appear in the mass spectrum, due to the low concentration of the compound

reference compounds confirmed that both of them contain unbranched alkyl moieties, i.e., they are dodecyl propenoate (**2**) and dodecyl propanoate (**3**).

The presence of a relatively large number of steroids, which eluted from the apolar column with retention times between approximately 87 and 105 min, in the femoral secretion of the sungazer suggested to us that these compounds could have a semiochemical function in this animal.

The interpretation of the mass spectra of steroids has been discussed in great detail (Budzikiewicz 1972; Thenot and Horning 1972; Shackleton 1985). Some of the more informative diagnostic ions in the mass spectra of the steroids identified in the femoral secretion of the sungazer and of their TMS derivatives are summarized in Table 2. Using this information and retention time comparison with authentic synthetic steroids, seven steroids were identified in the secretion. Constituent **46** was tentatively identified as 4,4-dimethyl-cholest-8-en-3β-ol. However, the published mass spectral data of this steroid did not correspond well with the mass spectrum of constituent **46**, and therefore, this compound remains unidentified. Another ten steroids could not be identified, some of which are also present and unidentified in other lizards (Martín and López 2006b).

The abundant steroids plus the well-known fixative squalene may function as the major controlled-release carrier materials in the secretion. Escobar et al. (2003) and Alberts (1990) postulated that cholesterol and the protein fraction could serve as controlled-release carrier materials or fixatives for semiochemicals in some lizards. It is also possible that the long-chain esters, hydroxy esters, and lactones (López and Martín 2005a, b, 2006; Martín and López 2006a, b) could have the same function in other lizard species. Lactones, long chain esters, and hydroxy esters were not found in the femoral gland secretion of the sungazer.

Squalene is the major component in the secretions collected from males during April 1998 and January 1999. In the secretions collected from males during October 1998, the component with the highest relative concentration differed among individuals and was squalene, cholest-5-en-3β-ol, or lanost-8-en-3β-ol. The major component in female secretions collected during these three seasons was usually either cholesterol or cholesta-5,7-dien-3β-ol, although in each of these seasons, a different female had octadecanoic acid as the major chemical.

Available information on the role of chemical communication in the social behavior of Cordylid lizard species is limited to a study of the behavior of the Cape girdled lizard, *Cordylus cordylus*, a member of the Cordilidae family, which showed that males and females can discriminate between their own secretions and those of individuals of the same sex (Cooper et al. 1999). Further research is needed to test whether *C. giganteus* individuals respond to femoral gland secretions of conspecifics and, if so, which (if any) of the chemicals identified herein mediate such responses.

Acknowledgements The research was supported by Stellenbosch University and the National Research Foundation, Pretoria, South Africa. Lanral Ruddock is thanked for the collection of secretions.

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acknowledged on the grounds of theoretical considerations and empirical demonstrations involving mainly visual- or acoustic-based communication systems (Maynard Smith and Harper 2003). Nevertheless, a growing body of evidence indicates that odors are broadly used in individual, sexual, and species recognition in vertebrates, and may be reliable signals of quality and compatibility (Wyatt 2003).

Birds were once considered to have a poor sense of smell, but more recent findings have modified the knowledge of this subject. Many birds species are sensitive to odors, and actually use olfaction in some behavioral tasks (for a review, see Roper 1999). However, compared to other organisms, information on the production or social use of self-produced odor compounds in birds has been largely neglected. Recent studies suggest that odor may play an important role in mate choice of birds (Zelano and Edwards 2002; Bonadonna and Nevitt 2004; Soini et al. 2007).

Procellariiform seabirds (petrels and albatrosses) have the most highly developed olfactory anatomy and neuroanatomy known in birds (Bang 1966). The first suggestion of the function of this anatomy was for finding food (Grubb 1972), and this was subsequently shown for many petrel species (reviewed by Roper 1999 and Nevitt and Bonadonna 2005a). Odors may also help petrels and albatrosses to navigate when flying across the oceans (Nevitt and Bonadonna 2005b).

Within species, petrels are faithful for life to their breeding colony and to their partner. In many species, pairs breed in underground burrows. Monogamous pairs lay a single egg, and partners alternate parental duties and foraging trips to sea (Warham 1996). Returning from the sea, many burrowing petrel species approach the colony and nest at night (to avoid avian predators, Mougeot and Bretagnolle 2000). Odors play a fundamental role in finding the burrow, and an odor signature of the nest facilitates burrow recognition in the dark (Bonadonna et al. 2003, 2004).

We recently showed that Antarctic prions, *Pachyptila desolata*, were able to recognize their own as well as their partner's odor, suggesting that personal odors may play a role in the nest's olfactory signature. This was the first evidence of individual recognition through odor in a bird species. Moreover, quite unexpectedly, we discovered a pattern of scent discrimination that is well-documented in mammals: Antarctic prions avoided self-odor when presented with the scent of another conspecific (Bonadonna and Nevitt 2004). Such behavior was first reported in mice, and was related to major histocompatibility complex genes (MHC), kin recognition, and inbreeding avoidance (Manning et al. 1992). This could also be the case in petrels, as high phylopatry may expose them to the risk of inbreeding and should select for mechanisms that allow for kin discrimination (Zelano and Edwards 2002).

Even after years of preservation, collected petrel feathers smell distinctive to the human nose (Bonadonna, personal observation). Birds protect and waterproof their feathers by preening them with the products of the uropygial gland (Stettenheim 1972). These products may carry odors that play a role in the behavior of birds (Soini et al. 2007). For example, odors produced by this gland play a role in sexual behavior of domestic ducks, *Anas platyrhynchos* (Balthazart and Schoffeniels 1979; Jacob et al. 1979). In petrels, the products of this gland may be species-specific (Jacob and Hoerschelmann 1982) and may constitute an olfactory signature on petrel feathers.

We hypothesized that feather scents of Antarctic prions constitute an individual olfactory signature, and we predicted that the scents produced by the feathers of the same bird in two different years should be more similar to each other than the scents produced by the feathers of two different birds in the same year. We report the results of our study herein.

Methods and Materials

Feather samples Feather samples were collected on Ile Verte (49°51′S, 70°05′E) in the Kerguelen Archipelago (southern Indian Ocean). The first sample was collected in the 2002–2003 austral summer from 10 pairs of ringed Antarctic prions, *P. desolata*, (20 birds), and again in the 2003–2004 and 2004–2005 austral summers (hereafter called years). We thus obtained feather samples for three consecutive years on seven females and six males (individuals may not survive and pairs may not nest every year, Warham 1996). Fifty to 200 mg of feathers were cut from the duvet around the uropygial gland with steel scissors cleaned in methanol between samples. Feathers were stored in aluminum paper, in a closed plastic bag, and refrigerated. Plastic bags were opened just before chemical analysis.

Chemical analysis Feathers of each sample were weighed and immersed in 4 ml dichloromethane for 24 hr. After this, the feathers were removed, and the solvent allowed to evaporate to 250 μl in a fume hood. Twenty microliters of *n*-dodecane (purity 99.8%, Sigma Aldrich Switzerland) in dichloromethane (200 μg/ml) were added to each extract as an internal standard. The samples were analyzed on a Varian CP3800 gas chromatograph (Varian, Palo Alto, CA, USA), equipped with a flame ionization detector and a Varian CP-Sil 5 CB Low Bleed MS (25 m×0.25 mm, ID, film thickness 0.25 μm) column. The oven was programmed as follows: 50°C for 2 min, 3°C min^{-1} to 100°C, 2.7°C min^{-1} to 140°C, 2.4°C min^{-1} to 170°C, and 10°C min^{-1} to 290°C. An on-column injector at 250°C was used with helium (1 ml min^{-1}) as carrier gas. To determine the identity of compounds, gas chromatography–mass spectrometry was employed using a Varian CP3800 coupled to a Varian Saturn 2000 ion trap mass spectrometer equipped with splitless injection and the same capillary column as above. The scan range of the ion trap was 38 to 350 *m/z*. The oven temperature program was the same as that above except the initial temperature of 50°C was held for 3 min. Total ion chromatograms and mass spectra were recorded in the electron impact ionization mode at 70 eV. Identification of compounds was based on mass spectral fragmentation pattern, using various libraries [Wiley 138 and NBS 75K libraries (McLafferty 1998), NIST98 (Stephen 1998), and the compilation by Adams (1995)], and chromatographic retention index (RI).

Selection of compounds and statistical analyses for comparison of chemical profiles We concentrated our analysis on compounds with retention times between 3 and 40 min in the FID chromatogram, on the basis that compounds with RI of greater than 1,700 on an apolar column do not have sufficient vapor pressure to be perceived by birds. Birds do not have a "wet" nose such as some mammals (e.g., dogs, mice, cats, etc) or a vomeronasal organ (Bang and Wenzel 1985) and, therefore, are likely only to perceive molecules that are borne by an air flow. From the 39 samples, we distinguished 99 different peaks; some were found in most samples, others in only a few. We considered each compound as a distinct dependent variable (99 variables) and each bird in each year (hereafter called bird/year) as a sampling unit (39 units). The data set contained a large number of potential dependent variables compared to the low number of sampling units. Therefore, we reduced the number of compounds (dependent variables) by using two different working hypotheses. Firstly, we hypothesized that the olfactory signature of birds is a bouquet composed of compounds present in all birds in different relative concentrations (Alberts 1992). Compounds of high occurrence (HOC) were selected as follows. We considered the third year profile of a bird (i.e., the profile obtained from the most recent feather sample). We used a compound only if it was present in the third year profile (i.e., the most recent feather sample) of at least 10 individual birds; 28 compounds fulfilled this criterion. Then, for all 13 birds and for each year, we considered the amount of these

compounds (amount=0 if compound was absent) and ran a principal component analysis (PCA; proc PRINCOMP in SAS 8.2) on the correlation matrix. This generated synthetic dependent variables that captured systematic associations among compounds.

Secondly, we hypothesized that the olfactory signature of birds is a bouquet composed of compounds present in a few birds, but with different association patterns, independent of compound concentration (Alberts 1992). Compounds of low occurrence (LOC) were selected as follows. We used a compound only if it was present in the third year profile of, at most, 5 individual birds; 21 compounds fulfilled this criterion. For all 13 birds and for each year, we ran a multiple correspondence analysis (MCA; proc CORRESP in SAS 8.2) on the contingency table to generate synthetic dependent variables that captured systematic presence/absence associations among compounds.

For each synthetic variable from PCA and MCA analyses, we estimated the variance among birds over years and the variance among years with respect to birds. These variances were accounted for in mixed effects linear models (proc MIXED in SAS 8.2) by the random factors "bird" and "year," respectively. An estimate of the residual variance was also provided. The measure of repeatability of the olfactory signature of an individual bird was computed as the ratio of the variance among birds consistent over years (i.e., "bird random effect") to total variance (intraclass correlation coefficient [ICC]). The blend of volatile compounds was considered as an individual olfactory signature if the variance component "bird" differed significantly from zero and if the ICC was higher than 50%. The fixed factor "sex" was also included in the models.

Results and Discussion

Representative chromatograms obtained from extracts of a single bird in each of the 3 yr are given (Fig. 1). The compounds identified in our analyses (Table 1) and used in our PCA and MCA were lipids. This is not surprising, considering that these compounds were probably derived from the uropygial gland, which principally produces waxes and greases to protect feathers (Jacob and Ziswiler 1982). Among the identified volatile compounds are fatty alcohols, fatty aldehydes, and hydrocarbons (Table 1).

Lipids are important as odors of vertebrates (Alberts 1992; Hagelin et al. 2003; Burger 2005), and some of the compounds we identified in Antarctic prions have already been identified as mammalian semiochemicals. For example, alkanes, such as decane and octane, are specific odor compounds from forehead hairs of white-tailed deer, *Odocoileus virginianus* (Gassett et al. 1997) and interdigital secretions of bontebok, *Damaliscus dorcas* (Burger et al. 1999). Aldehydes such as (*E*)-2-hexenal, dodecanal, and nonanal are present in urine of deer mouse, *Peromyscus maniculatus* (Ma et al. 1999) or in bontebok, (Burger et al. 1999), and alcohols such as dodecanol, heptanol, and tetradecanol are present in the preorbital secretion of grysbok, *Raphicerus melanotis* (Burger et al. 1996). Given the structure of these compounds and their function in other vertebrates, it seems likely that these compounds are produced endogenously through the oxidation or reduction of fatty acids. This is consistent with recent findings in other birds such as the dark-eyed junco, *Junco hyemalis* or green woodhoopoe, *Phoeniculus purpureus* (Burger et al. 2004; Soini et al. 2007).

Analysis of the variation in HOC The first three PCA components each accounted for a noticeable percentage (>25%) of the variation in the concentration of each of the 28 compounds selected. These three components represented variation in the concentration of 24, 3, and 1 distinct compounds and accounted for 76%, 8%, 5% of the total variation in

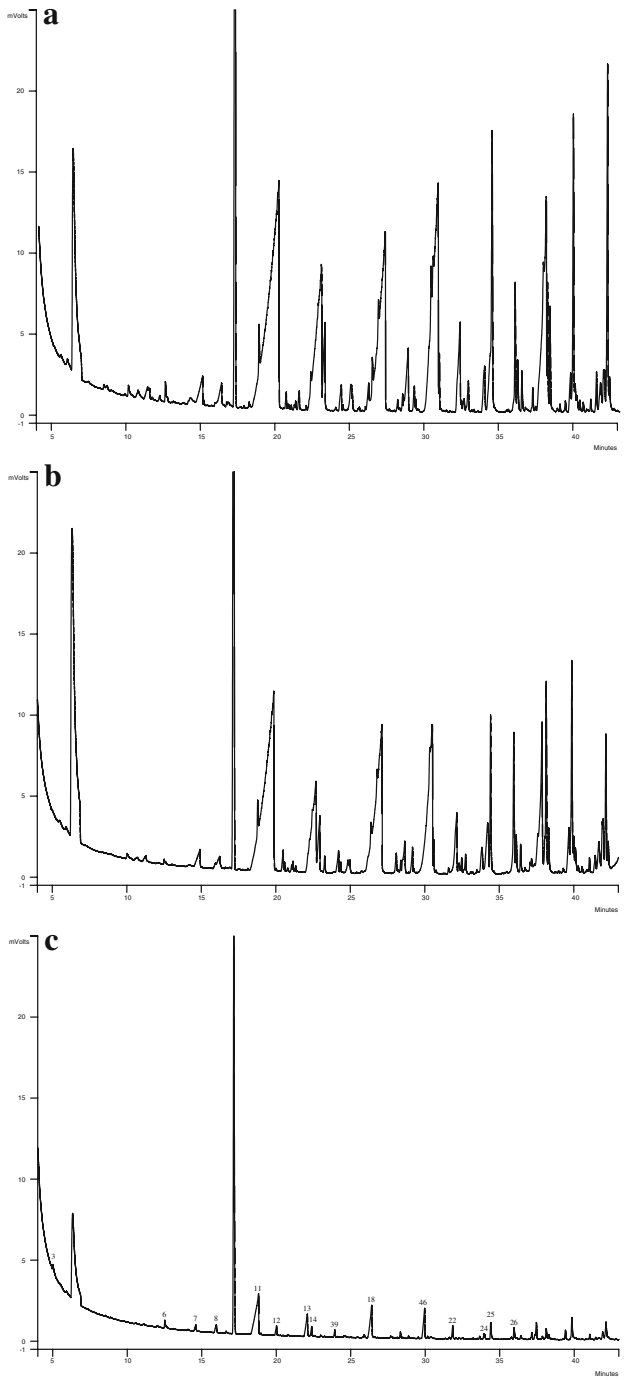


Fig. 1 Representative flame ionization detection chromatograms for Antarctic prion number KA26622. **a**, **b**, and **c** Chromatograms obtained with feathers of the first, second, and third year respectively. In the third year chromatogram, *numbers* on peaks refer to Table 1

Table 1 List of HOCs and LOCs used in analyses

No.	RI	Compounds	Analysis
1	760	NI	HOC
2	795	Hexanal	HOC
3	820	(*E*)-2-hexenal[a]	HOC
4	955	NI	HOC
5	1,073	Octanol	HOC
6	1,090	Nonanal	HOC
7	1,142	NI	HOC
8	1,174	Nonanol	HOC
9	1,190	Decanal	HOC
10	1,228	NI	HOC
11	1,235	4-Methyloctanoic acid	HOC
12	1,260	Nonanoic acid	HOC
13	1,315	3-Methylnonanoic acid	HOC
14	1,318	4-Methylnonanoic acid	HOC
15	1,327	(*Z*)-9-undecenal[a]	HOC
16	1,340	NI	HOC
17	1,361	Methyl nonanoate	HOC
18	1,418	4-Methyldecanoic acid	HOC
19	1,465	Dodecanol	HOC
20	1,500	Pentadecane	HOC
21	1,505	NI	HOC
22	1,550	Ethyl 3-methylundecanoate	HOC
23	1,596	4-Tetradecenal[a]	HOC
24	1,610	NI	HOC
25	1,625	3-Methyltridecanol	HOC
26	1,665	Tetradecanol	HOC
27	1,695	Pentadecanal	HOC
28	1,700	Heptadecane	HOC
29	730	NI	LOC
30	800	Octane	LOC
31	941	Heptanol	LOC
32	1,000	Decane	LOC
33	1,016	(*E*)-2-octenal[a]	LOC
34	1,050	NI	LOC
35	1,108	NI	LOC
36	1,121	Isododecane[a]	LOC
37	1,225	Isotridecane[a]	LOC
38	1,350	NI	LOC
39	1,380	Decanoic acid	LOC
40	1,395	Dodecanal	LOC
41	1,410	Isopentadecane[a]	LOC
42	1,455	NI	LOC
43	1,460	(*E*)-2-dodecenol[a]	LOC
44	1,495	Tridecanal	LOC
45	1,510	NI	LOC
46	1,513	3-Methylundecanoic acid	LOC
47	1,520	NI	LOC
48	1,595	Tetradecanal	LOC
49	1,690	Heptadecene[a]	LOC

No.: compound number, *RI*: retention index, *NI*: not identified.

[a] Tentative identification.

Table 2 Statistical modelling of the variation in 3 synthetic variables derived through PCA analysis from the variation in concentration of 28 HOCs

	Estimated Variance	SE	*P*
PCA first component (1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28)			
Sex			0.239
Year	2.193	3.714	0.277
Bird	0	Ne	1
Residual variance	19.568	4.678	
Intraclass correlation coefficient=0			
PCA second component (12, 16, 21)			
Sex			0.265
Year	0.050	0.154	0.373
Bird	1.020	0.635	0.054
Residual variance	1.316	0.380	
Intraclass correlation coefficient=0.43			
PCA third component (5)			
Sex			0.315
Year	0.042	0.140	0.382
Bird	0.049	0.230	0.416
Residual variance	1.239	0.358	
Intraclass correlation coefficient=0.04			

For the fixed effect, sex, the *P* value is that associated with an *F* test with 1 degree of freedom for the dependent variable and 24 residual degrees of freedom. For the random effects, year and bird, the *P* value is that associated to a Wald test.

The compounds for which the squared correlation coefficient R^2 with focal PCA component is >0.20 are given in parentheses.

SE: standard error, *Ne*: no standard error estimates could be produced for variance estimates equal to zero.

concentration of compounds, respectively. For these three synthetic variables, the variance estimates for the factors "bird" and "year" did not differ significantly from zero. The variation among birds over years accounted for a small fraction of the total variation with the possible exception of the second principal component (Table 2). It is possible that fatty acids present on feather samples collected in the first 2 yr continued to be modified during preservation, changing the relative quantity of derived "signature compounds". No sex effect was detected for any of the three synthetic variables.

According to these results, variation in the concentration of HOC was not likely to constitute an individual olfactory signature. However, we cannot exclude the possibility that our sampling protocol was not subtle enough to detect differences and that a possible signature of same compounds at different relative concentration exists. More sophisticated individual odor sampling protocols are needed to test this.

Analysis of the variation in LOC The first four MCA dimensions accounted for a substantial percentage (>20%) of the variation in the presence/absence of each of the 21 compounds selected. These four dimensions represented the variation in the presence/absence of 9, 4, 2, and 5 distinct compounds and accounted for 35%, 19%, 10%, and 8% of the total variation in the presence/absence of compounds, respectively. For the first two of these synthetic variables, variation among birds over years ("bird" factor) was statistically significant and accounted for a large fraction of the total variation (Table 3). Identified

Table 3 Statistical modelling of the variation in 4 synthetic variables derived through MCA analysis from the variation in presence/absence of 21 LOCs

	Estimated Variance	SE	*P*
MCA first dimension (29, 33, 34, 35, 36, 37, 46, 47)			
Sex			0.542
Year	0	0.015	0.490
Bird	0.323	0.163	0.024
Residual variance	0.169	0.0499	
Intraclass correlation coefficient=0.66			
MCA second dimension (30, 41, 43, 48)			
Sex			0.634
Year	0	Ne	1
Bird	0.188	0.107	0.040
Residual variance	0.178	0.050	
Intraclass correlation coefficient=0.51			
MCA third dimension (40, 49)			
Sex			0.051
Year	0	Ne	1
Bird	0	Ne	1
Residual variance	0.200	0.047	
Intraclass correlation coefficient=0			
MCA fourth dimension (31, 32, 38, 44, 45)			
Sex			0.716
Year	0.033	0.043	0.221
Bird	0.087	0.057	0.063
Residual variance	0.126	0.037	
Intraclass correlation coefficient=0.35			

For the fixed effect, sex, the *P* value is that associated to an *F* test with 1 for the dependent variable and 23 residual degrees of freedom. For the random effects, year and bird, the *P* value is that associated to a Wald test. The compounds for which the percent variation explained by the focal dimension is >20% are given in parentheses.

SE: standard error, *Ne*: no standard error estimates could be produced for variance estimates equal to zero.

compounds responsible for the statistical difference were isododecane, 3-methylundecanoic acid, (*E*)-2-octenal, and isotridecane for the first synthetic variable, and octane, isopentadecane, (*E*)-2-dodecenol, and tetradecanal for the second synthetic variable. Therefore, we believe that these LOC compounds may constitute an individual olfactory signature repeatable over years (ICC=0.66 and 0.51, respectively) that distinguishes individuals ("bird" effect *P*=0.024 and 0.04, respectively; Fig. 2).

The effect of the sex of the bird was close to being significant for the third synthetic variable (*P*=0.051; Table 3). However, in recent experiments in which odors of male and female birds were presented simultaneously in a Y-maze, neither male nor female Antarctic prions showed any preferences (Bonadonna et al. unpublished data). More work is needed to resolve this discrepancy.

In summary, our results, although preliminary and lacking direct behavioral confirmation for the compounds, suggest the existence of an endogenous individual olfactory signature in Antarctic prions, originating from the uropygial gland: an individual is more similar to itself with regard to volatile compounds, from year to year, than it is to a conspecific. This

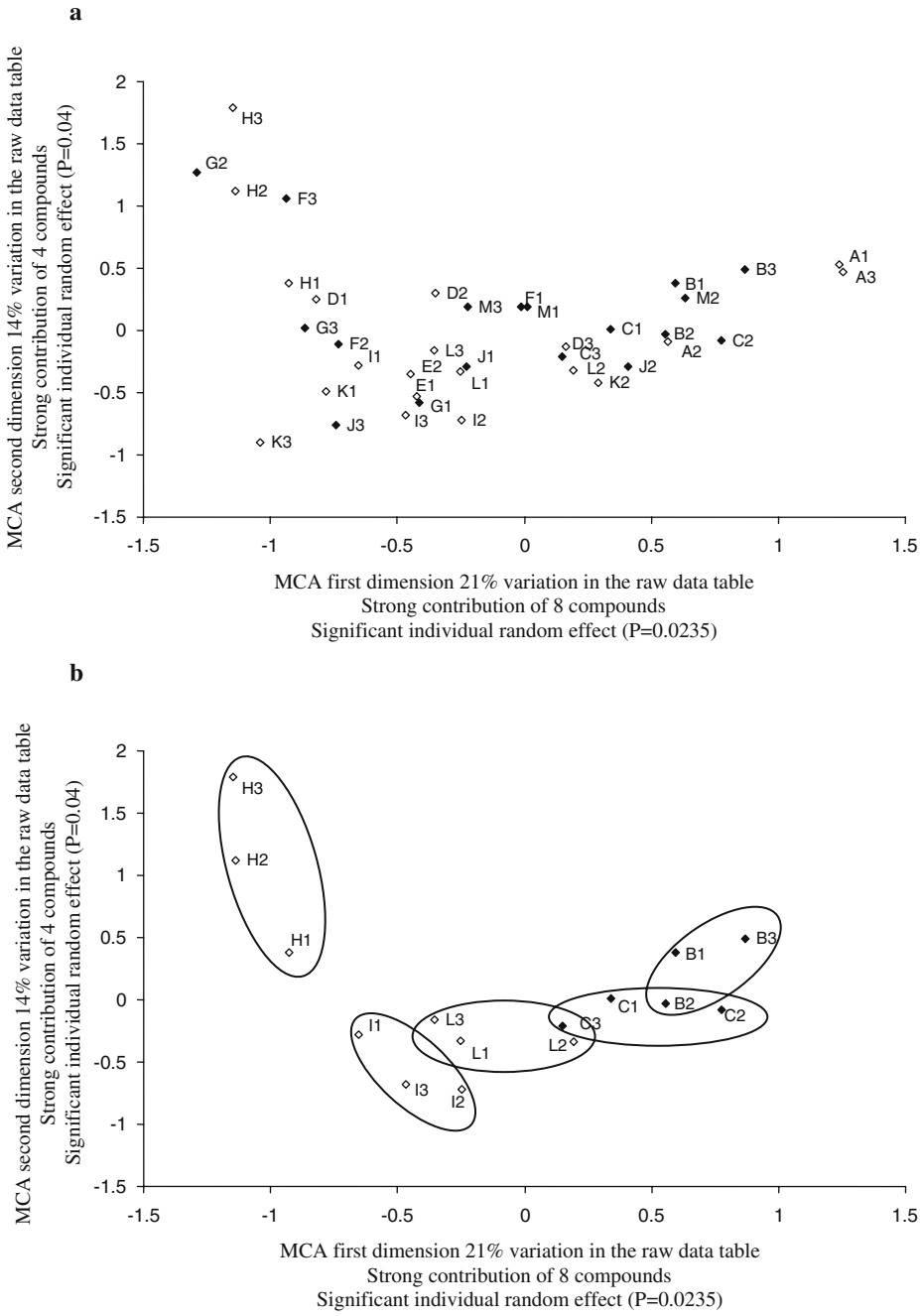


Fig. 2 **a** Volatile compound profile of 13 birds (*letter*) over 3 yr (*number*) as described by 2 synthetic variables derived through MCA analysis from the variation in the presence/absence of 21 volatile LOCs. *Open symbols* represent females; *filled symbols* represent males. **b** Same representation as in **a** but only selected birds are presented to better show clustering

is consistent with behavioral studies on partner-odor recognition (Bonadonna and Nevitt 2004). As this odor is likely endogenous, it might also broadcast the compatibility and quality of a potential mate as previously proposed (Zelano and Edwards 2002). To our knowledge, this is the first demonstration of a putative odor signature in birds.

Acknowledgments This study was supported by the Institut Polaire Français Paul Emile Victor (IPEV, Program no. 354) and performed in adherence to the IPEV/CNRS ethical guidelines. We thank B Buatois for the help with the gas chromatography; Dr. G Nevitt, C Bajzak, and Dr. S Caro for the help in the field; Prof. F S Dobson, Dr. P D'Ettorre, and Dr. M-C Anstett and two anonymous reviewers for the critical review of an earlier draft of the manuscript.

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three genotypes. Sinigrin, 4-hydroxyglucobrassicin, and phenylethylglucosinolate were present at significantly higher levels in flowers than in leaves. In response to caterpillar feeding, sinigrin levels in both leaves and flowers were significantly higher than in undamaged plants, whereas 4-hydroxyglucobrassicin leaf levels were lower. Our results show that feeding on flower tissues, containing higher concentrations of glucosinolates, provides *P. brassicae* with a nutritional benefit in terms of higher growth rate. This preference appears to be in contrast to published negative effects of volatile glucosinolate breakdown products on the closely related *Pieris rapae*.

Keywords Herbivory · Lepidoptera · Pieridae · Myrosinase · Isothiocyanates · Nutrition · *Brassica nigra*

Introduction

Most plants are not simply homogeneous resources to herbivorous insects but produce discrete organs with different functions. In addition to the vegetative leaf tissues, plants produce reproductive organs (flowers, seeds) that may exhibit significant differences in levels of both nutrients and defensive secondary metabolites. Specialized guilds of herbivores are known to exploit specific plant structures (Schoonhoven et al. 2005). Flowers and seeds may be attacked by insects that rarely feed on leaf tissues, and vice versa. Other herbivores exhibit more plasticity in food selection and will readily feed on all available parts of the plant. Even the latter herbivore types display some preference for a specific plant structure, presumably as a means of obtaining optimal nutrient intake, limiting competition, or obtaining enemy-free space.

The cabbage white butterflies *Pieris brassicae* L., *Pieris rapae* L., and *Pieris napi* L. are specialized on the plant family Brassicaceae. Interactions between these butterflies and their host plants have been explored in detail, and they have acquired the status of a model system in the field of insect–plant biology (Feltwell 1982; Chew and Renwick 1995; Renwick 2002). Glucosinolates, characteristic of the Brassicaceae, play a crucial role as chemical mediators of these interactions. Adult females of *P. brassicae* and *P. rapae*, as well as their larvae, exploit glucosinolates as token stimuli during selection of host plants for oviposition and feeding (Renwick et al. 1992; van Loon et al. 1992; Moyes et al. 2000; Schoonhoven and van Loon 2002). The majority of *Pieris–Brassica* interaction studies has focused on cultivated forms of *Brassica oleracea* L., which are biennials. Other wild crucifers, such as the annual black mustard, *Brassica nigra* L. (Koch), are reported to be host plants for *P. brassicae* in Europe as well (Harvey et al. 2003). We studied within-plant feeding site location in detail on three genotypes of *B. nigra*. We included the analysis of leaf and flower glucosinolates to assess whether a chemical basis for selection of either plant organ in terms of quality or quantity of these compounds could be determined. In addition, we investigated whether growth rates differed between caterpillars feeding on either leaves or flowers to assess whether the observed preference behavior would confer a nutritional benefit.

Methods and Materials

Plants Seeds of *B. nigra* var. *abyssinica* A. Braun from three early flowering accessions were obtained from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands).

The accessions were: A, CGN06619 (feral population, collected in 1975 from the Peloponesus, Greece); B, Junius CGN06618 (advanced cultivar, collected in 1977 in Germany); and C, Giebra CGN06620 (advanced cultivar, donated in 1965 by the Botanical Garden of the Justus Liebig University, Giessen, Germany). Sowing was done on April 7, 2004, in a greenhouse at 22±2°C and 60±5% RH. Two weeks later, plants were individually transplanted into 1.5-l pots and transferred to another greenhouse compartment on April 30, 2004. This compartment was kept at 22±2°C, 60% RH, natural light supplemented by additional illumination by 500-W SON-T lamps (Philips, Eindhoven, The Netherlands) for 16 hr per day. Flower bud formation and flower opening were recorded daily to determine developmental stage (Harper and Berkenkamp 1975).

Insects *Pieris brassicae* caterpillars were obtained from a laboratory strain established in 2004 and reared on Brussels sprouts (*B. oleracea* var. *gemmifera* cv Cyrus) plants grown in a climatized greenhouse, under the same conditions as given above for *B. nigra* plants. The colony was maintained in a climatized room at 22±1°C, RH 40±5%, and a photoperiod of L16:D8.

Caterpillar Position on the Plant On the first day of an experiment, newly hatched *P. brassicae* larvae were collected from the laboratory culture and taken to the greenhouse. Three caterpillars were placed on the first true leaf below the lowest flowering branch of a plant in growth stage 4.1 (Harper and Berkenkamp 1975). Total numbers of plants used from each accession were A, 8; B, 7; and C, 9. Caterpillar position on the plants was scored each morning in four categories: on a true leaf, on a small leaf in the inflorescence, on a flower, and on the stem. Caterpillar instar stage was recorded daily until the fifth instar was reached. In the late fifth instar, caterpillars that had entered the wandering phase left the plants.

Caterpillar Growth on Leaves and Flowers Three neonate caterpillars were inoculated on each of 20 plants of accession A, on a mature leaf just below the inflorescence. The inoculation took place when plants had just started to flower (growth stage 4.1). Just after caterpillars had molted to the third instar, they were transferred to the lowest branches of the inflorescence on half of the plants. We applied a specially constructed water barrier to prevent caterpillars from migrating from leaves to the inflorescence or vice versa, while not influencing the microclimate around the plant (Fig. 1). Fresh body weights of larvae were determined to the nearest milligram on a Mettler electronic balance at three time points separated by 3-d intervals.

Preparation of Plant Samples for Glucosinolate Analysis Ten seedlings of each of the three accessions were individually transplanted to 1 1/2-l pots. Plants were maintained at 22±2°C, RH 60±5%, during the 16-hr photophase (8 Philips SON-T Agro 400-W sodium lamps) and 8-hr scotophase.

At day 47 after sowing, leaf and flower samples were taken from all 10 plants of each accession. After sampling, five plants of each accession were designated untreated (controls). Three groups of 10 newly hatched *P. brassicae* caterpillars were placed on three leaves of the five treated plants. As soon as the caterpillars had migrated to the flowers, which took place late in the second or early in the third instar (days 5–6), leaves damaged by the caterpillars were sampled. Leaves of a similar age were sampled from the control plants and intact flowers were sampled from both control and treated plants.

The flower and leaf samples were kept in paper envelopes and stored in a −20°C freezer before and after freeze-drying. Samples were freeze-dried in the envelopes for 2 d (Labconco Freedry system). After freeze-drying, the envelopes were sealed in plastic and sent to the laboratory of NA in Denmark for chemical analysis.

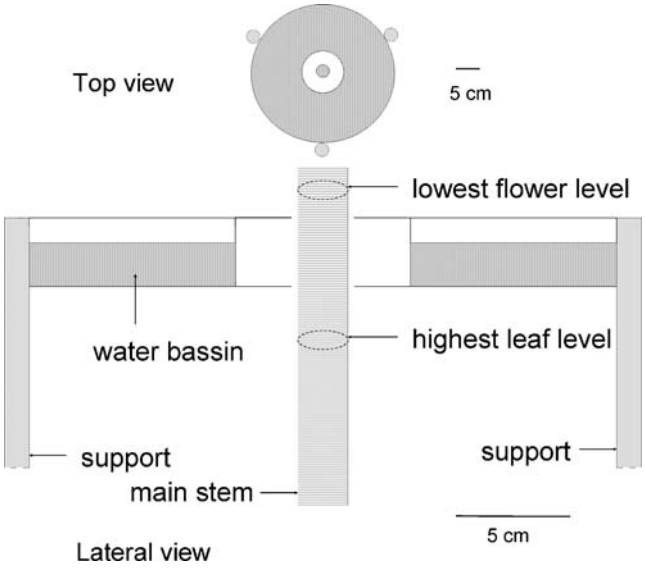


Fig. 1 Schematic drawing (top and lateral views) and dimensions of water barrier applied around the stem of *B. nigra* plants to prevent movement of caterpillars from leaves to flowers and vice versa. A circular water container was manufactured of plastic. The middle circle consisted of a central platform, preventing drowning of caterpillars feeding on the inflorescence in the rare events of falling down or downward migration. In the center of the platform, a hole allowed the main stem to grow through. The platform was put in place around the main stem just below the branch carrying the first flower buds. The circular opening between the central hole and the stem was blocked with soft foam (not drawn)

Determination of Glucosinolate Content Freeze-dried samples were weighed accurately into 10-ml centrifuge tubes (excluding the petiole part of leaves). For samples below 300 mg, the entire sample [27–257 mg dry weight (DW)] was used to avoid errors associated with subsampling, but from a few samples above 300 mg, a representative subsample was taken. Each sample was extracted 3× in 70% boiling aq. methanol with benzylglucosinolate as internal standard. The combined extracts were adjusted to 10.00 ml, of which an amount was applied to an anion exchange column (Agerbirk et al. 2001a). To keep within the linear range of the additional sample preparation steps, the amount of crude extract applied depended on the weight of the sample extracted as follows (DW extracted/volume applied): 25–75 mg/8 ml, 75–150 mg/4 ml, and 150–300 mg/2 ml. Glucosinolates were converted to desulfoglucosinolates (Agerbirk et al. 2007), which were eluted and quantified by high-performance liquid chromatography (HPLC) (Agerbirk et al. 2001a) and identified by comparison of retention times and diode array UV spectra with those of authentic standards (Agerbirk et al. 2001b). A number of unidentified trace peaks with areas typically less than 0.2% of the desulfosinigrin area were ignored. Generally accepted relative response factors were used for calculation of glucosinolate levels based on the HPLC peak areas (at 229 nm, band width 8 nm) of the desulfoderivatives (Wathelet et al. 2004). The recoveries of internal standard in the three extraction series were [mean (SD)] 99% (6%), 97% (13%), and 98% (4%), as compared to HPLC peak areas obtained after application of the internal standard only to DEAE columns in parallel control experiments. Preliminary experiments without the addition of internal standard (comprising both leaf and flower samples from plants both with and without caterpillars feeding) confirmed the absence of benzylglucosinolate as an endogenous glucosinolate in the material, in

accordance with previous reports (Feeny and Rosenberry 1982; Sang et al. 1984), allowing the use of this glucosinolate as internal standard. The identities of all desulfoglucosinolate peaks were confirmed by liquid chromatograph mass spectrometry (LC–MS) of representative samples with an Agilent 1100 Series LC (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). An XTerra MS C18 column (Waters, Milford, MA, USA; 3.5 μM, 2.1×100 mm) was used at a flow rate of 0.2 ml min^{-1}. The mobile phases were as follows: A, 0.1% (v/v) HCOOH and 50 μM NaCl; B, 0.1% (v/v) HCOOH and 80% (v/v) MeCN. The gradient program was 0 to 4 min, isocratic 2% (v/v) B; 4 to 10 min, linear gradient 2 to 8% B; 10 to 30 min, linear gradient 8% to 50% (v/v) B; 30 to 35 min, linear gradient 50 to 100% (v/v) B; 35 to 40 min, isocratic 100% B. The mass spectrometer was run in electrospray mode, observing positive ions. Mass spectral data were treated with the native DataAnalysis software.

The extraction procedure was somewhat simplified compared to the standard protocol (Agerbirk et al. 2001a, originally optimized for seed extraction), as variable sample weights were accepted, and mechanical homogenization during extraction was avoided. These simplifications were justified by results of initial control experiments: A number of both leaf and flower samples of varying weights (up to 400 mg) were first subjected to the extraction described above. Then, the residue was further extracted for another three times, but this time with mechanical homogenization. In all cases, low relative amounts of glucosinolates (1–16% compared to the first extract) were detected in the extract obtained with mechanical homogenization (with a tendency for increasing percentage with increasing sample weight). Proportional amounts of internal standard were similarly recovered in all the extracts obtained with mechanical homogenization, showing that the internal standard had already been completely mixed with endogenous analytes during the extraction without homogenization. Hence, it was concluded that the ratio of internal standard to endogenous glucosinolates extracted without mechanical homogenization was representative of their initial concentrations.

Statistical Analyses Proportion of days caterpillars spent on different positions on the plant was treated as binomial data. Distributions over the four positions were compared by using the Kolmogorov–Smirnoff test (GenStat release 8.11; Anonymous 2005). Caterpillar weights were analyzed by analysis of variance. The amount (μmol/g DW) of each glucosinolate detected in the leaf and flower samples were analyzed with a generalized linear model after a logarithmic transformation. The effect of time (before and during caterpillar feeding), treatment (undamaged and damaged by caterpillars), plant tissue (leaf and flower), and genotype (accessions) and all possible two- and three-way interactions were investigated (GenStat release 8.11).

Results

Position on the Plant First and second instars fed primarily on leaves, whereas third instars migrated via the small leaves of the flower branches to the flower buds and flowers. Once flower feeding began, no further leaf feeding was observed (Fig. 2). The distribution of caterpillars over the four positions was statistically similar for the three genotypes (Kolmogorov–Smirnoff test, $P>0.05$).

Caterpillars fed in discrete meals two to three times each hour, interspersed with phases without feeding. On a leaf, after termination of a meal, they moved away from the freshly

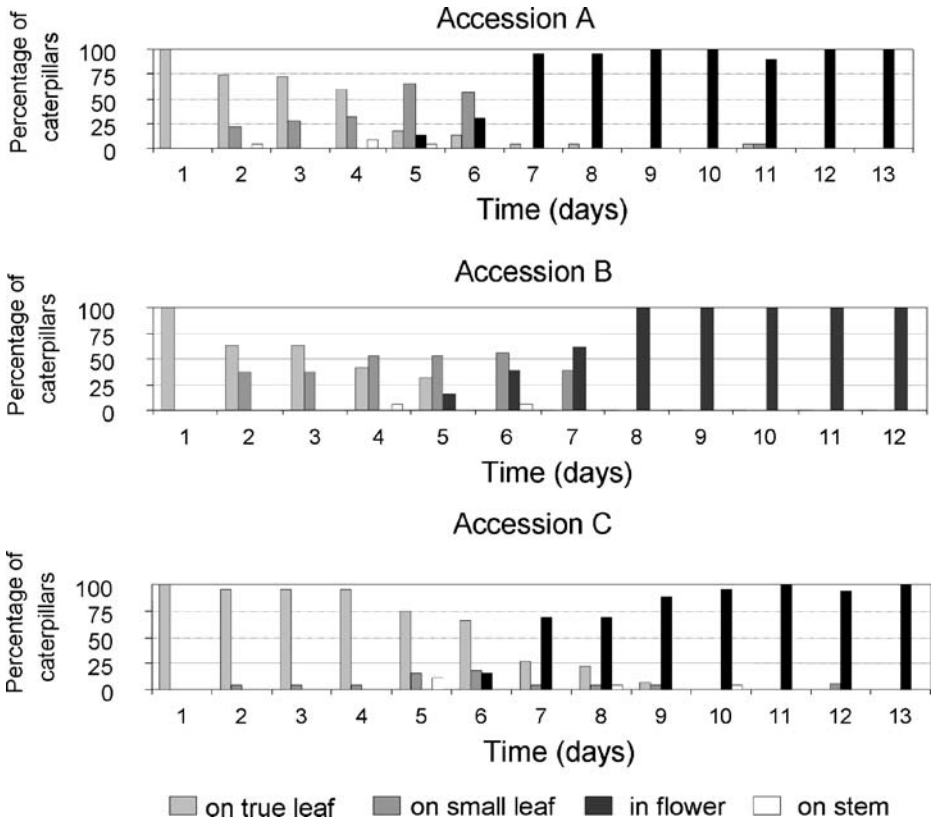


Fig. 2 Frequency distribution of caterpillar position on *B. nigra* plants observed daily over a period of 12–13 d. On day 1, neonates were introduced on a true leaf just below the inflorescence. Four organ positions were distinguished: on a true leaf, on a small leaf in the inflorescence, on a flower, or on a stem in the inflorescence. Percentages are averaged based on three caterpillars per plant for eight, seven, and nine plants of accessions A, B, and C, respectively

damaged wound, after which they rested most of the time elsewhere on the leaf until they moved back to the feeding site. By contrast, in the inflorescence, during a single meal, caterpillars moved from flower to flower along flowering branches. A fifth-instar caterpillar consumed on average 135±21 buds and flowers. When they were not feeding, *P. brassicae* larvae rested on stems immediately adjacent to the flowers.

Caterpillar Growth Caterpillars feeding on flowers of accession A since molting to the third instar had significantly higher fresh body weights on each of the three measuring points (mid-L4 and early and mid-L5 and late L5; Fig. 3) than those that were confined to leaves ($P<0.01$, generalized linear model).

Glucosinolate Levels Five glucosinolates were identified: the aliphatic allylglucosinolate (sinigrin), which occurred in large amounts, and low levels of four aromatic glucosinolates including 2-phenylethylglucosinolate and the three indole glucosinolates indol-3-ylmethyl-glucosinolate (glucobrassicin), 4-methoxyglucobrassicin, and 4-hydroxyglucobrassicin. Despite the minor levels of the identified aromatic glucosinolates, they were quantified

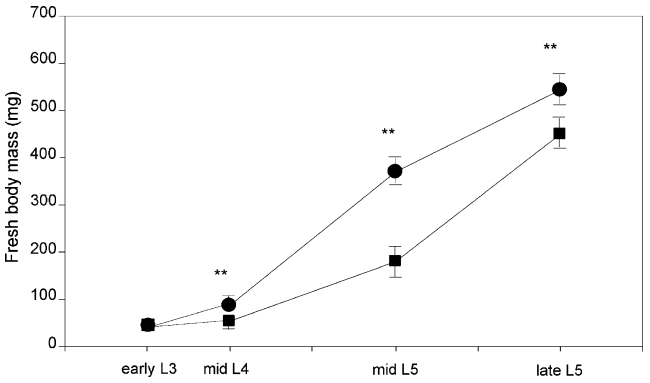


Fig. 3 Fresh body mass of *P. brassicae* on either flowers (*circles*) or leaves (*squares*) of *B. nigra* in mid-fourth, mid-fifth, and late-fifth instar developing on *B. nigra*, accession A. Mean and SEM are plotted for 30 caterpillars

and included in the statistical analyses because of their well established chemosensory influences on *Pieris* species (Schoonhoven and van Loon 2002). No other significant peaks were detected. 2-Phenylethylglucosinolate was either below the detection limit in leaves (accession C) or present at very low levels (range 0.001–0.013 μmol/ g DW). We carefully distinguished the peak with the exact retention time of (desulfo) 2-phenylethylglucosinolate from an unidentified trace peak that was more intense in leaf chromatograms (estimated levels 0.03–0.35 μmol/g DW in leaves, 0.01–0.08 μmol/g DW in flowers), that had a retention time 0.7 min longer than that of (desulfo) 2-phenylethylglucosinolate, and that did not coelute with the authentic standard of 2-phenylethylglucosinolate after spiking.

Genotype and tissue type were the most important factors affecting the levels of identified glucosinolates, and these factors showed a significant interaction (Table 1). No significant interactions were found between treatment and tissue ($P>0.05$ for all glucosinolates). Glucosinolate concentrations have been graphically depicted for accession A in Fig. 4. Sinigrin was by far the most abundant compound in all three genotypes, with levels at least 44 times higher (range 44–305) in leaves and at least 87 times higher (range

Table 1 Results from generalized linear model analysis on log-transformed data of glucosinolate concentrations in two tissue types (leaves and flowers) for three genotypes of *B. nigra*

Factor	Sinigrin	PE	GB	4OHGB	4MeOGB
Time	ns	ns	ns	***	ns
Tissue	***	***	ns	***	ns
Treatment	*	ns	ns	*	ns
Genotype	***	***	***	***	ns
Tissue × genotype	**	***	ns	*	ns

Treatment means presence or absence of third-instar caterpillars feeding on flowers during 4 d. Effect of the factor "time" refers to changes over 4 d in intact plants

PE = phenyethylglucosinolate; GB = glucobrassicin; 4OHGB = 4-hydroxyglucobrassicin; 4MeOGB = 4-methoxyglucobrassicin

*$P<0.05$; **$P<0.01$; ***$P<0.001$

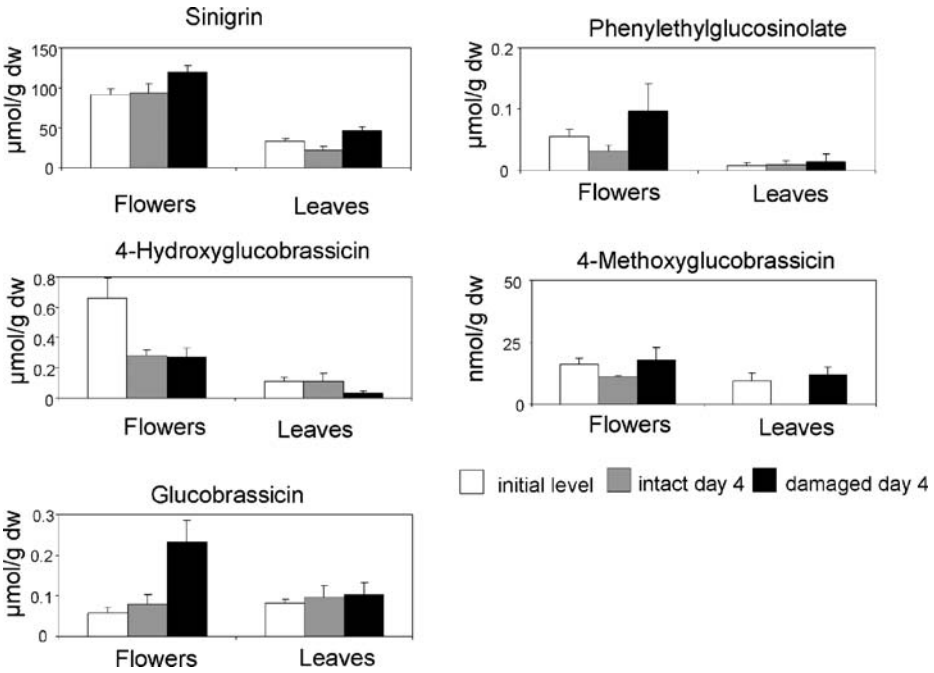


Fig. 4 Concentrations of five glucosinolates (mean + SEM) in leaves and flowers of 10 plants of accession A. For both leaves and flowers, the initial concentration just before treatment, the concentration in leaves or flowers of plants damaged by feeding caterpillars during 4 d, and the concentration determined in leaves or flowers of intact plants 4 d after caterpillars were introduced on the treated plants

87–447) in flowers of any of the three genotypes and treatments than the second most abundant compound, 4-hydroxyglucobrassicin. Sinigrin, 4-hydroxyglucobrassicin, and phenylethylglucosinolate were present at significantly higher levels in flowers than in leaves. Sinigrin levels were ca. fivefold higher in flowers than in leaves. In response to caterpillar feeding, sinigrin levels in leaves were higher than in undamaged plants ($P<0.05$; Table 1). In flowers of accession A (Fig. 4), there was a similar tendency for induction of sinigrin by caterpillar feeding, but such induction was not seen with flowers from the other two genotypes (results not shown). Levels of other glucosinolates showed moderate variations that were not affected by the experimental variables (Table 1, Fig. 4 and results not shown). There was no systematic induction of any indole glucosinolate or of total indole glucosinolates in response to caterpillar feeding (results not shown).

Discussion

Our data show that *P. brassicae* third, fourth, and fifth instars preferentially feed on *B. nigra* flowers. Preference was absolute: although leaf material was available in surplus, caterpillars fed exclusively on flowers. Historically, *P. brassicae* has been commonly considered a folivore (Feltwell 1982), with its impact on plants examined primarily from this perspective. To the best of our knowledge, this is the first detailed study showing that *P. brassicae* larvae preferentially feed on the buds and flowers of its host plant. The status of

P. brassicae as an agricultural pest has invariably resulted in a focus on cultivated, biennial forms of *B. oleracea* in studies of its host plant relationships. We also observed flower and silique feeding under field conditions (Smallegange et al., unpublished results).

The impact of folivores on plants has both direct and indirect effects. Directly, these herbivores reduce the leaf area available for photosynthesis, thereby decreasing the plants' ability to garner resources for defense and reproduction (Marquis 1984; Mothershead and Marquis 2000; Schoonhoven et al. 2005). Indirectly, leaf herbivory can delay flowering (Meyer and Root 1993; Strauss et al. 1996) or can alter developing flowers both physically (Strauss et al. 1996; Lehtilä and Strauss 1999; Mothershead and Marquis 2000) and chemically (Lohman et al. 1996; Karban and Baldwin 1997). This may cause a reduction in plant attractiveness to pollinators or in the efficiency of pollen delivery, resulting in decreased pollination and, subsequently, reduction of seed set and overall fitness of the plant. Florivory, feeding on flowers, has received less attention than folivory or feeding on other vegetative plant parts despite its common occurrence and potentially high impact on plant fitness (recently reviewed by McCall and Irwin 2006). Feeding on flowers causes damage to structures essential for plant reproductive output (Juenger and Bergelson 1997; Adler et al. 2001). Indirectly, petal damage leads to a reduction in pollinator visitation that not only reduces reproductive success but also affects male fitness (via pollen removal) of the plant as well (Karban and Strauss 1993; Krupnick and Weis 1998; Adler et al. 2001). However, the compensatory abilities of plants can partially overcome the impact of floral herbivory (Hendrix 1988; Krupnick and Weis 1998). Removal of reproductive organs as a preferred mode of feeding has evolutionary consequences, as this type of attack is likely to exert a stronger selection pressure than leaf feeding, especially in annual species like *B. nigra*. Although many plant species accumulate higher concentrations of defensive secondary metabolites in reproductive organs than in vegetative organs (Schoonhoven et al. 2005), in *B. nigra*, these higher levels are not sufficient to deter the specialist feeder *P. brassicae*. A putative barrier to florivory by generalist herbivores lies in the higher quantities of secondary plant compounds that are typically found in these organs relative to the levels reported for leaves (Rask et al. 2000; Fahey et al. 2001; Brown et al. 2003; Strauss et al. 2004; this study). The location of high concentrations of defensive secondary plant compounds, including glucosinolates, in reproductive structures is consistent with the optimal distribution of chemical defenses predicted by plant defense theory (Zangerl and Bazzaz 1992; van Dam et al. 1996; Wallace and Eigenbrode 2002; Strauss et al. 2004). Myrosinases, β-thioglucosidase enzymes that convert glucosinolates into products such as isothiocyanates and nitriles, which are volatile in the case of, e.g., sinigrin and 2-phenylethylglucosinolate, occur in different forms in flowers and leaves (Rask et al. 2000). By using headspace techniques, isothiocyanates and nitriles have been reported from mechanically macerated bud samples of several *Brassica* species (Tollsten and Bergström 1988). Phenylacetonitrile was detected in headspace samples of intact *B. rapa* L. flowers (Omura et al. 1999). Benzylcyanide was released in significantly higher amounts from *Brassica napus* L. in midflowering than in bud stage (Jönsson et al. 2005). High levels of isothiocyanates are potentially toxic to both specialist and generalist herbivorous insects (e.g., Li et al. 2000; Agrawal and Kurashige 2003), while even low levels may be detected by specialized sensory receptors and assist specialized herbivores in host plant finding (Renwick et al. 2006).

The growth rate we observed for caterpillars feeding on flowers was remarkable. The feeding site preference of L3–L5 caterpillars presents a case of within-plant selective foraging, the adaptive value of which might be the ingestion of higher nutritional content in flowers, known to act as nutrient sinks, compared to leaves. Nutrient acquisition during the

larval stage can affect pupal survival, longevity, and fecundity of Lepidoptera (Feltwell 1982; Van der Meijden et al. 1984; Metspalu et al. 2003). For example, female pupae of *Tyria jacobaeae* were significantly larger when caterpillars were reared on leaves and inflorescences of *Senecio jacobaea* plants than when reared on leaves only. This is advantageous because large pupae have a higher eclosion success, and it may explain why *T. jacobaeae* females prefer flowering plants for oviposition (Vrieling and de Boer 1999).

Faster growth has often been interpreted as a mortality-reducing factor. It is assumed that by reducing the amount of time that larvae are early instars, when they are most vulnerable to attack by natural enemies, they will experience higher survival (the 'slow-growth–high-mortality hypothesis' sensu Clancy and Price 1987; Benrey and Denno 1997). This has been experimentally confirmed under field conditions for the interaction between *P. rapae* feeding on *B. oleracea* and its predators and parasitoids such as *Cladophora glomerata* (Loader and Damman 1991; Benrey and Denno 1997). However, for other tritrophic interactions, faster growth was associated with higher mortality due to natural enemies (e.g., Clancy and Price 1987). Biere et al. (2002) found that larvae of the moth *Hadena bicruris* exhibit a strong preference for seeds of their food plant, *Silena latifolia*, over leaf tissues. Herbivore development proceeded more rapidly on seeds, and the larvae were less susceptible to parasitism from their main endoparasitoid, *Microplitis tristis*.

Herbivorous insects specialized on glucosinolate-containing plants typically avoid the formation of toxic isothiocyanates by employing specialized detoxifying mechanisms. In the case of *P. brassicae*, this is accomplished by a nitrile specifier protein (NSP) in the gut that changes the products of the myrosinase-catalysed hydrolysis of glucosinolates from isothiocyanates to relatively harmless nitriles (Wittstock et al. 2004), which may be further metabolized before excretion depending on side chain structure (Agerbirk et al. 2006; 2007). Shortly before this mechanism was reported, it was discovered that allyl isothiocyanate, the volatile hydrolysis product of sinigrin, is toxic to the specialist feeder *P. rapae* when feeding on an artificial diet (Agrawal and Kurashige 2003). Assuming a 20% DW in the artificial diet as used by Agrawal and Kurashige (2003), the maximum concentration to which *P. rapae* caterpillars were exposed in their study was 8.5 μmol/g DW. This dose caused significant negative effects on growth rate and inhibited development. In contrast, sinigrin levels in *B. nigra* flowers were more than 10 times higher (Fig. 4) and flowers sustained higher growth rates of *P. brassicae* than leaves, which had average levels of sinigrin two times higher than the corresponding amount of allylisothiocyanate as applied in the artificial diet. Assuming that isothiocyanates were produced in significant amounts during ingestion of glucosinolate-containing foliage by *P. rapae*, an involvement of the glucosinolate–myrosinase system in defense against this specialist species was suggested. *Brassica nigra* plants that had been induced by previous *P. rapae* feeding damage had a 27% increase in sinigrin concentration, but also 43% higher trichome density (Traw and Dawson 2002). Our data confirm the reported induction of sinigrin in leaves upon feeding, but also demonstrate that the magnitude of the induction is minor relative to the difference between leaves and flowers. With the elucidation of the NSP-based mechanism for detoxification of the glucosinolate–myrosinase system, the suggested involvement of the glucosinolate–myrosinase system and defense to specialists now needs to be re-evaluated. Formation of isothiocyanates is to be expected only if the myrosinase activity in the plant is so high that available NSP cannot completely direct the reaction towards nitriles, combined with glucosinolate levels sufficiently high to produce toxic levels of isothiocyanates. The selective foraging on flowers by *P. brassicae* documented here demonstrates that the caterpillars are able to detoxify even high levels of sinigrin, such as those present in *B. nigra* flower tissue. Not only high glucosinolate levels but also high myrosinase activity would be needed to exert toxic effects on *Pieris*

caterpillars, as has been experimentally demonstrated for another glucosinolate-adapted herbivorous insect, *Plutella xylostella* (Li et al. 2000). Glucosinolates occur in cells of all organs of Brassicaceous plants, whereas myrosinases can be found in scattered glucosinolate-free cells. It remains to be examined whether flower buds of different age, flowers, and siliques of *B. nigra* have cells that contain myrosinases. In the related *B. napus* L., expression of the MYR1-gene coding for particular myrosinase isoforms has been demonstrated to occur in all organs investigated, including petals (Falk et al. 1992), although myrosinase was absent in very young flower buds in this species (Andréasson et al. 2001). Myrosinase gene promoters fused to beta-glucuronidase as a reporter showed expression of myrosinase in idioblast myrosin cells in immature and mature seeds and phloem myrosin cells of *B. napus* (Thangstad et al. 2004). Data on headspace composition of inflorescences actually fed upon by *Pieris* spp. are not available. Follow-up studies on the types and quantities of volatile breakdown products emitted by herbivore-damaged flowers are required to quantify myrosinase activity levels in flowers. If these levels are low, the better performance associated with flower feeding might be reconciled with a role of the glucosinolate–myrosinase system in defense against the specialist *P. brassicae*. However, a likely and simple explanation of the better performance associated with flower-feeding may be that the caterpillars are able to efficiently detoxify the glucosinolate–myrosinase defense system in both organs, and that flowers, major nutrient sinks on a plant, are more nutritious than leaves. The fact that early instars did not migrate to flowers may be an adaptation to factors other than nutrient content: leaves are the site of oviposition, are present earlier in the season than flowers, and may provide better refuge from natural enemies for small instars. We have never observed *P. brassicae* or *P. rapae* ovipositing on flowers or flowering stems.

Neither *P. rapae* nor *P. brassicae* sequester glucosinolates for their own defense against natural enemies (Müller et al. 2003), in contrast to what had been published earlier by Aplin et al. (1975). The intensely green, clear regurgitant these caterpillars instantly, and typically, produce when attacked may contain highly concentrated glucosinolate hydrolysis products (nitriles) stored in the foregut. Presence of concentrated glucosinolate hydrolysis products may explain the obvious repellent effect of regurgitant to natural enemies such as *Cotesia* wasps and predators (Brodeur et al. 1996). Consequently, caterpillars may prefer flower tissues that contain high levels of these compounds, thus boosting their defense against both specialist and generalist natural enemies (Soler et al. 2005). Behavioral experiments with parasitoids and predators known to act as common natural enemies of *P. brassicae* (Feltwell 1982) are required to test this hypothesis.

Acknowledgments The authors thank André Gidding, Frans van Aggelen, and Leo Koopman for rearing *P. brassicae*, Unifarm for sowing and planting *B. nigra*, and the CGN (Wageningen, The Netherlands) for supplying *B. nigra* seeds. Matthieu Joosten (Laboratory of Phytopathology, Wageningen University) is acknowledged for freeze-drying the samples. We thank C.E. Olsen for LC–MS analyses and Birgitte B. Rasmussen and Lene K. Petersen for excellent technical assistance. Financial support from Gluds Legat and the Dutch Science Foundation NWO (grant 811.34.006) is gratefully acknowledged.

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Keywords *Solanum tuberosum* · Plant volatiles · Induced response · Methyl jasmonate · Solanaceae · Tritrophic interaction · *Phthorimaea operculella* · *Orius insidiosus*

Introduction

In response to herbivore damage, plants synthesize and release complex blends of volatile compounds that serve as indirect defenses that mediate the attraction of predators and/or parasitoids of the herbivores responsible for the damage (Vet and Dicke 1992; De Moraes et al. 1998; Kessler and Baldwin 2001; Turlings and Wackers 2004; de Boer et al. 2004). Herbivory is not the only factor that induced the release of plant volatiles, even deposition of eggs by herbivorous arthropods may induce volatile attractants (Colazza et al. 2004; Hilker and Meiners 2006).

By releasing volatile compounds after herbivore attack, a plant can affect both predation and oviposition rates, thus influencing control over its herbivore population (Kessler and Baldwin 2001). Volatile compounds may also act as a direct defense by repelling herbivores (Turlings and Wackers 2004, and references therein). However, for some herbivores, plant volatiles represent a complex message that is interpreted according to the biological context, and these compounds may also attract other herbivores and thereby incur ecological costs (Kessler and Baldwin 2002; Heil 2004; Bengtsson et al. 2006; Hori et al. 2006, Srinivasan et al. 2006).

Plant volatiles include isoprenoids, carotenoids, benzenoids, phenylpropanoids, fatty acid derivatives, and amino acid derivatives (Turlings et al. 1998; Ferry et al. 2004; Dudareva et al. 2006). Herbivore feeding and mechanical damage can induce responses in plants that may be either systemic or restricted to the wounded site (Potting et al. 1995). Emission of plant volatiles in response to herbivores, resulting in the attraction of parasitoids and predators, has been documented in many systems, most of which include crop species (Turlings and Wackers 2004).

The potato tuberworm moth, *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae), is a major pest in many potato-producing countries. At plant senescence, adult females lay eggs in soil cracks and on exposed tubers (Traynier 1975; Radcliffe 1982). After eclosion, the larvae mine into tubers, leaves, and stems, resulting in significant yield losses (Fenemore 1988). The highest percentage of infestation is found in exposed tubers above ground. This infestation is also a major source of further infestation in storage (Sileshi and Teriessa 2001). Until recently, control of this pest has been achieved with insecticides (Coll et al. 2000), semiochemicals, and natural enemies (Salehi and Keller 2002; Saour 2004). Less attention has been paid to the mechanisms of host-plant location and the tritrophic interactions mediated by compounds released by the plant.

Minute pirate bugs of the genus *Orius* (Hemiptera: Anthocoridae) are important natural enemies of pest insects and mites (Bush et al. 1993), and they are used as biological control agents in many parts of the world (Butler and O'Neil 2007 and references therein). Our field observations in potato crops of Southeastern Brazil show that adults of *O. insidiosus* attack and consume eggs and early instar larvae of *P. operculella* in partially exposed tubers and leaves of potato plants. Preliminary studies on the response of *P. operculella* to potato plants showed that these organisms did not respond to volatiles from the aerial portion of these plants in olfactometer bioassays. We then focused our studies on the behavioral responses of *P. operculella* to exposed potato tubers, to address the question of whether these insects are able to adjust their response to volatiles emitted from tubers with respect to

the tuber induction status. The role of volatiles for the polyphagous predator *Orius insidiosus* (Say) was also examined.

Methods and Materials

Insects and Plant Material Larvae of the potato tuberworm moth *P. operculella* were collected from potato fields at Paranapanema, Sao Paulo State, Brazil. Insects were reared on potato tubers inside plastic containers at 25±5°C and 60±10% relative humidity on a 12:12 hr L/D photoperiod regime. Pupae were sexed and placed in Petri dishes until adults emerged. Adults were maintained in net cages (20×20×20 cm) under the same conditions as larvae and fed with a 10% (*v*/*v*) honey solution. To avoid inbreeding, new larvae were collected regularly from potato fields. To obtain mated females, one newly emerged female and two males were confined together in plastic containers (5 cm high×10 cm diameter) for 5 d. Only females of *P. operculella* were used. Adults of the minute pirate bug *Orius insidiosus* were collected from flowers of *Helianthus annuus* and *Bidens pilosa* (Asteraceae) adjacent to potato fields with high infestation by the potato tuberworm moth, and kept in 10 ml glass vials under same conditions described above. Tubers of *Solanum tuberosum* L. (Solanaceae) cultivar Agata were obtained from the *Instituto Agronômico* (IAC), Campinas, São Paulo state, Brazil.

Olfactometer Bioassays To study the behavioral response of mated and unmated females of *P. operculella* and adults of *O. insidiosus* toward induced and noninduced volatiles of exposed tubers of potato plants, we conducted four-arm olfactometer bioassays (Vet et al. 1983) with the following three treatments:

Treatment 1: one tuber with approximately 400 g was mechanically damaged three times with a wheel cutter (1 cm diam.) over a distance of 3 cm, to mimic the damage caused by the first instars of *P. operculella*; *Treatment 2*: a tuber damaged by 50 first instars of *P. operculella*, which were allowed to feed before testing; *Treatment 3*: one intact tuber.

The bioassays were conducted after 24 hr of damage, to allow tuber penetration by small first instars of *P. operculella* in treatment 2. Each trial consisted of a combination of three treatment sources offered to the insects (mechanical damage, larval damage, and intact), whereas one chamber was kept empty. Treatments were randomly assigned to the arms.

The insects to be tested were introduced individually through the air extraction hole, after which the airflow was immediately started. The olfactometer was rotated every five trials to avoid positional biases not associated with the experimental treatment. After each trial, the apparatus was cleaned with water, and a four-empty-arm bioassay was conducted to evaluate the presence of volatile residuals. The tuber in the chambers was changed after cleaning. The response of each insect was tested for a period of 5 min. Bioassays with *P. operculella* were conducted at night, whereas those with *O. insidiosus* were conducted with fluorescent light, according to the activity pattern known for these insects (Fenemore 1988; Reid and Lampman 1989). The insects were deprived of food for 24 hr before the bioassays. The insect response to volatiles was measured with two variables: (1) the percentage of time spent walking in each odor field, and (2) the final position, which was defined as the odor field where the insect was positioned at the end of the 5-min-period. The total number of insects tested for each bioassay varied from 40 to 60. All treatments were carried out in a room at 25±5°C and 60±10% relative humidity.

Collection and Analysis of Headspace Volatiles Collection of volatiles from tubers was performed by using three different sampling techniques: (1) a push–pull dynamic headspace collection; (2) a static headspace collection that used solid phase microextraction (SPME) (Tholl et al. 2006); and (3) a direct headspace sampling (Millar and Sims 1998). For the pull headspace sampling, the tubers (~400 g) were placed in a glass chamber (20 cm tall× 10 cm diam). Clean humidified air was forced through the chamber, regulated by a flowmeter at 500 ml min^{-1} positioned at the lower part of the chamber. Volatiles were trapped in a glass tube (7.5×0.5 cm) attached to the top of the glass chamber containing 100 mg Super Q adsorbent (80/100 Mesh, Alltech Assoc., IL, USA). The trap was connected to the outlet of the chamber and via another flowmeter to the vacuum compressor. Volatiles were eluted with 100 µl dichloromethane, and 1 µl eluate was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS). Empty chambers were used as a control to extract air volatiles in the three sampling techniques. SPME sampling technique was conducted with a SPME fiber coated with 100 µm of polydimethylsiloxane (Supelco, Bellefonte, PA, USA). The fiber was conditioned for 1 hr in a GC injection port at 250°C and afterward, placed inside the glass chamber above the tubers. After sampling, the fiber was analyzed immediately by GC-MS. Direct headspace sampling was conducted by removing an aliquot of 10 µl of headspace of the plant material placed inside the glass chamber with a gas-tight syringe and injected directly into the GC-MS. All collections were conducted at 25±3°C and 60±10% relative humidity. Each treatment was repeated five times.

Volatiles were characterized by GC-MS-EI on a Hewlett Packard 6890 gas chromatograph system with a fused capillary column (30 m×0.25 mm×0.25 µm, HP-5MS, crossbond 5%-phenyl-95%-dimethylpolysiloxane) directly coupled to a selective mass detector Hewlett Packard 5973. Injector and detector temperatures were 250°C and 280°C, respectively. Injections were made in splitless mode with a constant column flow of 35 cm sec^{-1} helium. The oven was programmed from 35°C min^{-1}, 3°C min^{-1} to 250°C for 40 min. Quantification of methyl jasmonate (MeJA) (see below) was calculated by constructing a calibration curve with known concentrations of synthetic standards, and correlating their peak areas with those of the samples collected from tubers.

Response of Insects to Synthetic MeJA To investigate if the biased preference of mated females of *P. operculella* and *O. insidiosus* to volatiles from undamaged and damaged potato tubers (see "Results") could be explained by differences in the concentration of MeJA, behavioral bioassays were conducted in the four-arm olfactometer with synthetic MeJA 95% [Sigma-Aldrich Co., Steinheim, Germany—a mixture of methyl jasmonate (95%) and two unknown isomers] placed on a rubber septum at the equivalent amount found in the direct headspace sampling of intact tubers (0.001 ng g^{-1} of fresh tuber). A behavioral response curve was built by using as reference the equivalent amount of MeJA (0.001 ng g^{-1}) plus two dilutions and five concentrations with a factor of three (0.0001, 0.0003, 0.001, 0.003, 0.009, 0.027, 0.081, and 0.241 ng g^{-1}). The response to each concentration was analyzed in one chamber of the olfactometer, whereas the other chambers were kept empty, as described by Vet et al. (1983). Treatments were randomly assigned to the arms. The response of the insects was measured by the time spent walking in each odor field.

Statistics As data on percentage of time spent walking in each odor treatment were not normally distributed in the bioassays with tubers, they were analyzed with a nonparametric Friedman test with a multiple comparison analysis for ranked data based on Nemenyi test

(Zar 1999). The number of final positions for each odor treatment was analyzed by using the Cochran *Q* test; pair-wise comparisons of the number of final positions were analyzed with a test for multiple contrasts of data subjected to the Cochran test (Zar 1999). Mean differences of the amount of MeJA among the treatments were analyzed with a one-way analysis of variance (ANOVA). A two-way ANOVA was used to analyze the response (mean time spent in each test odor field) of mated females of *P. operculella* and adults of *O. insidiosus* to synthetic MeJA, with insect species and MeJA concentration as fixed effects. Multiple comparisons were made by using a Tukey HSD test (Zar 1999).

Results

The response of females of *P. operculella* to volatiles of exposed potato tubers was dependent on their mating status and on the induction treatments. For unmated females, the percentage of time spent walking in the different fields failed to show a significant preference for any of the treatments (Friedman test: $\chi^2=5.57$, $df=3$, $P=0.135$). In contrast, mated females showed differences among the treatments (Friedman test: $\chi^2=13.87$, $df=3$, $P=0.003$), preferring to stay longer in the field corresponding to the treatment with intact tubers than in those containing tubers with mechanical damage, larval damage, or clean air (Fig. 1). The number of final positions followed the same pattern, with unmated females showing no significant results and mated females choosing to remain in the field corresponding to intact tubers significantly more than in the other treatments (Table 1).

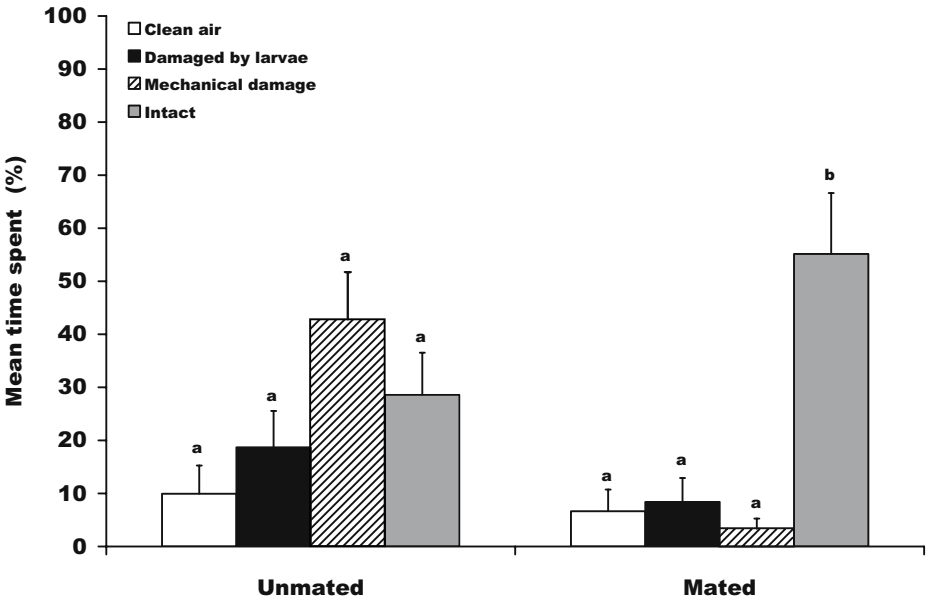


Fig. 1 Response of mated females of *Phthorimaea operculella* to volatiles from differently treated potato tubers. The mean percentages of time spent (±SE) within each field of the olfactometer were recorded for 5 min. Different letters indicate significant differences, evaluated by a multiple comparison test of ranked data based on the Nemenyi test ($P<0.05$)

Table 1 Total number of final positions of *Phthorimaea operculella* females and adults of *Orius insidiosus* to four different odor sources in the olfactometer

Treatments	Insects		
	Unmated *P. operculella*	Mated *P. operculella*	*O. insidiosus*
Clean air	2^a	3^a	1^a
Intact	6^a	11^b	3^a
Mechanically damaged	8^a	3^a	1^a
Damaged by larvae	4^a	1^a	14^b
Cochran Q test	Q=5.61; *N*=40; *df*=3, *P*=0.1320	Q=13.11; *N*=40; *df*=3, *P*=0.004	Q=22.00, *N*=40; *df*=3, *P*<0.001

Different letters in each row indicate significant differences by multiple contrasts for data subjected to the Cochran *Q* test ($P<0.05$, Zar 1999)

Adults of *O. insidiosus* also responded to the volatiles emanating from exposed tubers. The percent of time spent walking was significantly different among the treatments, with the insects staying longer in the field with tubers damaged by larvae than in those with intact or mechanically damaged tubers, or with clean air (Friedman test: $\chi^2=12.12$, $df=3$, $P=0.007$, Fig. 2). The same result was obtained for the number of final positions, which was significantly different among treatments showing that insects responded to volatiles from tubers infested with *P. operculella* larvae over those of intact and mechanically damaged tubers, or clean air (Table 1).

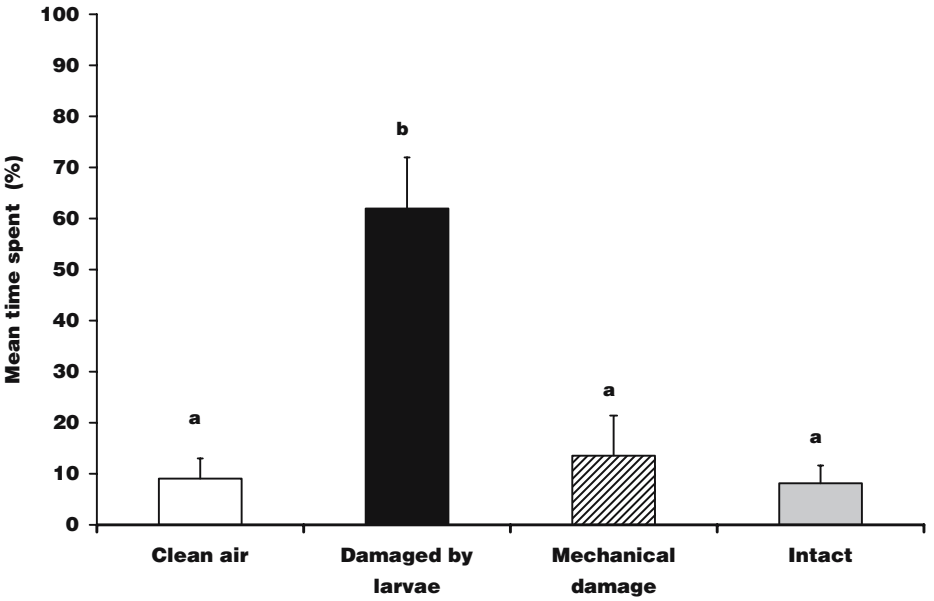


Fig. 2 Response of adults of *Orius indisiosus* to volatiles from differently treated potato tubers. The mean percentages of time spent (±SE) within each field of the olfactometer were recorded for 5 min. Different letters indicate significant differences evaluated by a multiple comparison test of ranked data based on the Nemenyi test ($P<0.05$)

No peaks were detected with either pull or static headspace SPME collection. The analysis of direct headspace of intact and induced tubers showed a mixture of isomers of MeJA [methyl jasmonate (90%) and two unknown isomers (10%)] as the only compounds. Confirmation of this result was made by co-injection with commercially supplied MeJA. MeJA measurements of the headspace from potato tubers were calculated by using a standard curve with five replicates and known amounts of synthetic MeJA injected into the GC-MS. Quantitative significant differences of the mean amounts of MeJA were found among treatments (one-way ANOVA: $F_{2,\ 9}=6.66$; $P=0.017$). Infestation with *P. operculella* larvae for 24 hr resulted in a six- to tenfold increase in the amount of MeJA emitted (0.090 ± 0.006 ng g^{-1} of potato fresh tuber) when compared with tubers damaged artificially (0.002 ± 0.0007 ng g^{-1}) (Tukey test: $P=0.042$), and intact tubers (0.001 ± 0.0003 ng g^{-1}) ($P=0.021$), respectively. There were no differences between the amount of MeJA emitted by intact or artificially damaged tubers ($P=0.890$).

Mated females of *P. operculella* and adults of *O. insidiosus* showed their highest activity on a different range of concentrations of synthetic MeJA (Fig. 3), and the response to MeJA was significantly different between insect species (two-way ANOVA: $F_{1,\ 281}=3.90$; $P=0.040$) and MeJA concentrations (two-way ANOVA: $F_{7,\ 281}=2.62$; $P=0.012$). Moreover, an interaction between species and MeJA concentrations was found (two-way ANOVA: $F_{7,\ 281}=5.53$; $P<0.001$). Adults of *O. insidiosus* showed a stronger response to higher concentrations of MeJA than mated females of *P. operculella*. Maximum range activity of mated moths and the minute pirate bug to synthetic MeJA was observed

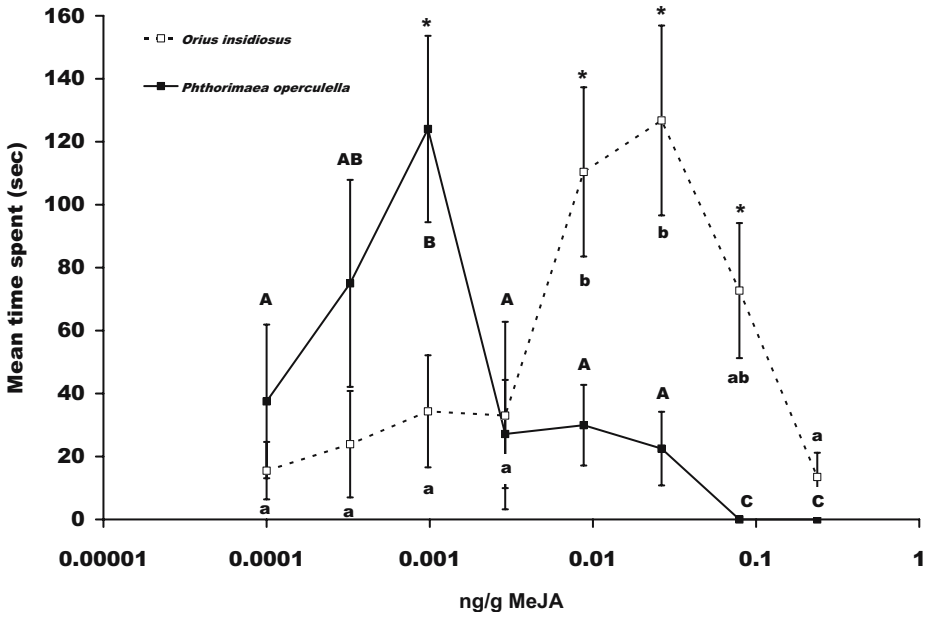


Fig. 3 Activity of mated females of *Phthorimaea operculella* and adults of *Orius insidiosus* to different concentrations of synthetic MeJA. The mean time spent (±SE) within each field of the olfactometer was recorded for 5 min. Differences were analyzed by using a two-way ANOVA. Different letters within each species indicate significant differences (Tukey test; $P<0.05$) among concentrations, being uppercase for *P. operculella* and lowercase for *O. insidiosus*. Asterisks indicate significant differences of each concentration between species (Tukey test; $P<0.05$)

at 0.0003 to 0.001 and at 0.01 to 0.05 ng g^{-1}, respectively, which correspond to the concentrations of intact and infested tubers detected in the direct headspace samplings.

Discussion

When allowed to select between potato tuber volatiles, mated females of *P. operculella* preferred the intact tubers over damaged ones, whereas unmated females showed no response. Several studies have shown that herbivores that forage for suitable food items often use volatiles emitted by host plants as cues (Prokopy and Roitberg 2001, and references therein). The decision to approach, feed on, or oviposit on a plant might depend on: (1) the plant's defense status, (2) the activity of conspecifics, and (3) the mating status of the animal. Thus, the ecological role of these compounds may differ among plant and herbivore species (Bolter et al. 1997; De Moraes et al. 2001; Rojas et al. 2003; Pickett et al. 2003; Heil 2004; Meiners et al. 2005; Tasin et al. 2005; Dickens 2006). The internal state of an animal, such as reproductive stage, also influences its foraging behavior (see Vet and Dicke 1992, for a review). Mated *P. operculella* females might use volatiles from intact tubers to locate a suitable site for oviposition. Several other insect species preferentially oviposit on undamaged rather than damaged host plants in response to volatile cues (De Moraes et al. 2001; Carroll et al. 2006) as at high infestation levels, volatiles released by damaged tubers may indicate: (1) herbivore population densities are already high, and as a result competition among larvae may be high (Prokopy and Roitberg 2001, and references therein); (2) the presence of natural enemies attracted by the volatiles, such as egg parasitoids responding to locally and systemically induced plant volatiles (Meiners and Hilker 1997; Lou et al. 2005); (3) direct plant defenses have already been activated; and (4) a lower nutritional value for their offspring than on intact plants (Bernasconi et al. 1998). Avoidance behavior mediated by induced plant volatiles also has been shown for some herbivorous insects (Meiners et al. 2005; Mehrnejad and Copland 2006). Thus, the effect of induced responses of potato tubers on populations of *P. operculella* and the sensory responsiveness of the females of this insect should be further investigated.

Plants responding to herbivore attack may release large amounts of volatiles into the environment. These play important roles in host location for many predators and parasitoids (De Moraes et al. 1998; Kessler and Baldwin 2001; Lou et al. 2005; Mehrnejad and Copland 2006; Yan and Wang 2006). Some species of *Orius* use plant volatiles in prey-searching behavior (Reid and Lampman 1989; VanLaerhoven et al. 2000; Tillman 2006, Mochizuki and Yano 2007), and our results indicate that *O. insidiosus* can exploit volatiles released by exposed potato tubers infested with larvae in the trophic system considered. The orientation of *O. insidiosus* to plant volatiles induced by larvae-infested tubers supports the hypothesis of Vet and Dicke (1992), who suggested that the ability of natural enemies to distinguish between intact and herbivore-induced plant synomones could increase their searching efficiency.

Regarding the role of MeJA as a mediator of tritrophic interactions, this compound has been found to occur constitutively in some plants and also to be inducible. Amounts of MeJA released by the plant are known to increase according to the extent of the damage (Preston et al. 2004). MeJA is well-known as a phytohormone signaling wounding (e.g., De Bruxelles and Roberts 2001; Howe 2004) and mediating even morphological changes (e.g., Boughton et al. 2005). A direct role of MeJA as cue for predators and parasitoids has not been well described (James 2005; James and Grasswitz 2005). However, our results suggest that it may mediate potato tuber attractiveness to herbivores and their natural enemies. At specific concentrations, MeJA mediates host recognition of the second and

third trophic levels on exposed potato tubers, by influencing *P. operculella* mated females to avoid previously attacked tubers, and by attracting the generalist predator *O. insidiosus* to tubers attacked by *P. operculella* larvae. In addition, the range of active concentrations corresponds with the respective emissions from intact and infested tubers found in headspace samplings.

Volatiles of different cultivars of potato plants attract potato pests (Bolter et al. 1997; Eigenbrode et al. 2002). Nevertheless, apart from being specific for the damaging herbivore species, herbivore-induced synomones may also be plant-specific and cultivar-specific (Takabayashi et al. 1991). To our knowledge, this is the first study of volatiles emitted by infested or intact potato tubers that mediate the interactions between herbivorous insects and their natural enemies.

Based on the results obtained in the laboratory study, we propose the following scenario for tritrophic interactions among potato plants, *P. operculella*, and the polyphagous predator *O. insidiosus*: Mated females of the potato tuberworm moth locate their host and lay egg clutches on intact tubers, which are partially exposed from the soil in older plants. The response of *P. operculella* and *O. insidiosus* to potato tubers is then modulated by MeJA. Mated females may locate a suitable site of oviposition by detecting the low concentrations of this compound emitted by intact tubers. Once the tubers are subjected to feeding by first-instar larvae, the plants release higher amounts of MeJA, thus becoming less attractive to mated tuberworm moth females, but attractive to the polyphagous predator *O. insidiosus*. Both *P. operculella* and *O. insidiosus* might have adapted to associate plant volatiles with the potential presence of suitable host plants or prey. Furthermore, the concentration of MeJA might determine the kind of behavioral response of both the herbivorous and the predatory species.

In South America, cultural practices, such as the type of irrigation and removal of the halum, are known to affect the level of tuber infestation by the potato tubermoth because they affect the exposure of tubers (CIP 1988). Proper implementation of control measures, such as filling soil cracks, affect the ability of adults of the potato tuberworm to oviposit on exposed tubers (Coll et al. 2000). Predation by *O. insidiosus* is restricted to eggs and the first instars of many insects; thus, it may be possible to use *O. insidiosus* against *P. operculella* in biological control programs, and the current study offers evidence to support the potential use of induced volatiles of potato tubers to improve the foraging efficacy of the minute pirate bug.

Acknowledgments We thank Ted Turlings and two anonymous reviewers for the valuable comments on the manuscript. We also thank Johana Rincones for reviewing the grammar and syntax of the English text. This work was supported by FAPESP (Fundação de Amparo à Pesquisa no Estado de São Paulo), grants number 04/12365-4 (AA), 98/01065-7 (JRT), and 01/06587-6 (JMSB).

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grasshoppers and Mormon crickets. A typical bait formulation consists of wheat bran or other solid carrier impregnated with an insecticide. Adding attractants and phagostimulants could result in greater bait acceptance, lure susceptible species to bait particles, and enhance palatability. Such additions to bait formulation make bait treatment more efficacious (Latchininsky and VanDyke 2006).

Vegetable oils have kairomonal attractant properties toward grasshoppers primarily due to the presence of linoleic and linolenic fatty acids (Bomar and Lockwood 1994a). These fatty acids are dietary essentials for grasshoppers and, once volatilized, can be detected by the insects' olfactory receptors (Bomar and Lockwood 1994b). Furthermore, fatty acids may be token stimuli for necrophagic grasshoppers seeking other nutrients, such as proteins, which are abundant in insect cadavers. Due to the presence of these fatty acids, certain vegetable oils used as insecticide carriers can function as "liquid baits" and markedly enhance the efficacy of grasshopper control programs (Lockwood et al. 2001).

The goal of this study was to develop a sensitive and efficient laboratory bioassay method and to apply this approach to identify particular vegetable oils that induce grasshopper attraction and feeding. Such oils may be useful kairomonal adjuvants and/or carriers for acridicide formulations.

Materials and Methods

Fatty Acid Analysis The initial selection of oils for screening was based on considerations of chemical profile, availability to pest managers, and cost. We sought oils with a fatty acid profile similar to that of the grasshopper *Melanoplus sanguinipes* (F.) (Barlow 1964), a common rangeland pest grasshopper, and that were widely available and inexpensive. A literature search and market investigation yielded eight candidate vegetable oils: canola, corn, linseed or flax, olive, peanut, rapeseed, soybean, and sunflower (Table 1). Subsequently, samples of these oils were obtained from available suppliers and analyzed for their actual fatty acid composition as described in Broughton and Wade (2002; Table 2). Lipids were extracted sequentially with chloroform-methanol (1:2, *v/v*), chloroform-saline (1:1, *v/v*), and one part chloroform (2×) following the method of Bligh and Dyer (1959). Chloroform fractions were pooled, evaporated to dryness, and suspended in minimal quantities of chloroform. After extraction, lipids were saponified in toluene and 0.5 mol/l KOH in methanol (8 min at 86°C)

Table 1 Typical fatty acid percent composition of some common vegetable oils (White 2000) as compared to *Melanoplus sanguinipes* adult grasshoppers

Fatty Acid Oil	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Other	Total
Canola	4	2	64	19	9	2	100
Corn	12	2	28	57	1	0	100
Flax*	5	5	20	16	53	1	100
Olive	14	3	71	10	1	1	100
Peanut	12	3	47	32	0	6	100
Rapeseed	3	1	24	15	7	50	100
Soybean	11	4	23	53	8	1	100
Sunflower	7	5	19	68	1	0	100
*Melanoplus sanguinipes***	11	4	19	20	43	3	100

*Values from Panford and deMan (1990)

**Values from Barlow (1964)

Table 2 Fatty acid composition (% weight) of some common vegetable oils from available sources analyzed at the university of wyoming nutrition laboratory, and percent similarity to fatty acid composition of *Melanoplus sanguinipes* adult grasshoppers

Vegetable Oil	Fatty Acid Source	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Other**	Percent Similarity
Canola	Puritan®	5	2	65	20	6	2	54
Corn	Safeway®	13	2	25	59	1	0	53
Flax	Rhône-Poulenc	5	4	19	14	58	0	85
Olive	Bertolli® Extra Virgin Oil	2	3	80	12	1	2	39
Peanut	Planters®	13	3	52	29	0	3	56
Rapeseed	Rhône-Poulenc	6	1	63	20	9	1	56
Rapeseed+Flax	Rhône-Poulenc	6	3	41	17	33	0	71
Soybean	Safeway®	12	5	25	51	7	0	65
Sunflower	Wesson®	8	5	15	72	0	0	47
*Melanoplus sanguinipes**	NA	11	4	19	20	43	3	100

*Values from Barlow (1964)

**Small amounts of fatty acids like Myristic, Palmitoleic, and Erucic

to liberate free fatty acids, cooled on ice, and acidified with 0.7 mol/l HCl in methanol. Free fatty acids were extracted with hexane (×2), evaporated to dryness, and methylated with ethereal diazomethane. The methyl esters were redissolved in hexane and analyzed by gas chromatography [Hewlett-Packard 5890 gas chromatograph with a DB23 capillary column (0.25 mm× 30 m, J & W Chromatography, Folsom, CA, USA) with hydrogen as the carrier gas]. Fatty acids were quantified by using pentadecanoic acid (NuCheck Prep, Elysian, MN, USA) as an internal standard.

Fatty acid profiles of the oils did not agree exactly with previously published data (Table 1). This is not surprising as fatty acids produced in any one type of oilseed may vary widely with geographic location, soil type, climate, agricultural practices, and even exposure to pesticides (White 2000).

Comparisons among the fatty acid profiles of the vegetable oils and *M. sanguinipes* were based on percent similarity (Kotila 1986). Furthermore, three fatty acids were of particular interest—linoleic, linolenic, and oleic—because they (mostly the first two and, to a lesser extent, the third) are the primary olfactory attractants of grasshoppers (Bomar and Lockwood 1994a, b). Consequently, the oils were ranked according to (1) percent similarity of their fatty acid profiles with *M. sanguinipes*; (2) combined, linolenic and linoleic acid content; and (3) oleic acid content. The ranking of each oil reflected the theoretical expectations of the oil performance as phagostimulants based on previous studies. The final ranking of vegetable oils as grasshopper attractants and feeding stimulants was developed after three sets of laboratory bioassays were performed with the selected oils.

Bioassay The non-diapausing strain of *M. sanguinipes* was selected for laboratory experiments. It is one of the few grasshopper species continuously available in laboratory colonies in the USA, and is a significant component of the rangeland grasshopper pest community (Pfadt 1988). In addition, its fatty acid profile is known (Barlow 1964).

Assays 1 and 3 were conducted on fourth instar nymphs. Nymphs were used in bioassays because the nymphal stage is when control programs generally should be conducted on

rangeland grasshoppers, fourth instar nymphs can be handled without injury, and feeding behavior is more consistent and reliable than in fifth instars (Bomar and Lockwood 1991). Nymphs were raised from eggs provided by the USDA-ARS Northern Plains Agricultural Research Laboratory in Sidney, Montana. Grasshoppers were hatched and reared according to Henry (1985). Assay 2 was conducted on adult insects, with equal numbers of males and females.

Pairs of grasshopper nymphs were used in assays, rather than single individuals. Adult grasshoppers were tested with just one individual per assay chamber. Before testing, all grasshoppers were held without food or water for a period of 4 h. Gladware® brand storage containers (739 ml capacity) were selected as an optimal assay chamber.

Bioassays were developed using a 24-h time period from introduction of the grasshopper(s) to the test chamber until scoring of the results. This amount of time was the minimum necessary to assure that grasshoppers had passed through an entire circadian cycle, an important consideration in context of their feeding periodicity (Lockwood et al. 1996). A longer duration of assay increased the probability that the nymphs would molt into the next instar, thereby invalidating the results. Environmental conditions were standardized at 27±1° C, 14:10 h light/dark, and 30% RH.

Whatman 1 filter paper (55 mm diam) was chosen for assays because it provided a neutral and stable substrate following Bomar and Lockwood (1994a, b). Oils were applied to flat filter paper discs with an airbrush applicator as described by Lockwood et al. (2001). This method allowed oil to be deposited at a rate that simulated aerial field applications of 1 l/ha (0.2 µl per disc) and 10 l/ha (2 µl per disc). However, the airbrush method of applications was not optimal because the oil was evenly applied and subsequent grasshopper feeding on was highly dispersed and not always evident. Often, nymphs would simply scrape the surface of the paper, rather than remove a readily discernible portion of the substrate. This made accurate scoring of the paper feeding difficult.

As an alternative method, the paper discs were folded in half to create a tent-like shape, which made it easy for the insects to grasp and feed on the edges. At each end of the crease, we applied various amounts of canola oil (from 0.1 to10 µl) until we found a volume that would create a visible spot (ca. 1 cm diam) without bleeding over the entire disc surface. A 2 µl drop created a distinct semicircular area for feeding, and in preliminary tests with 20 pairs of grasshopper nymphs, 60% of them removed a detectable portion of the oil-treated paper from the folded disc. In some cases, the nymphs chewed the entire semicircle of oil-impregnated paper, leaving behind only the untreated substrate. The 4 µl of oil per 55-mm filter paper is 20 times greater than the average amount that would be applied to the same surface area via most ultra low volume (ULV) aerial applications (1 l/ha is a typical rangeland ULV rate), but the application method provided an optimal "target" for grasshopper feeding in context of the assay.

A simple binomial approach was used to score insect feeding: Either a detectable (by unaided human vision) portion of the substrate was removed, or it was not. Efforts to generate more refined estimates for substrate removal or to determine whether there was surface scraping eroded the interobserver reliability of scoring.

Previous studies suggested that adding a water source during a feeding assay may decrease grasshoppers' physiological stress and increase their feeding on tested substrates (Bomar and Lockwood 1994a, b). As such, we conducted preliminary tests with a water-soaked cellulose sponges (ca. 3 cm^3) placed in the assay chambers. The oils in this test included 5 vegetable oils (canola, olive, rapeseed, flax, and a 1:1 blend of rapeseed and flax) and crop oil (a paraffinic oil as positive control) by using a pair of 2 µl drops. Data were analyzed by Pearson's chi-square linear-by-linear association with SPSS 14.0 statistical software. Results showed that tests with no water were generally more sensitive to differences among oils

(Table 3). Grasshopper feeding on different oils increase inconsistently, and only in two cases (rapeseed and canola) did the presence of water significantly ($P<0.05$) increase feeding (Table 3). Given that the inclusion of a water-soaked sponge added logistic complexity (i.e., keeping the paper from being wetted), raised humidity to unnaturally high levels in the chamber, provided an alternative, confounding substrate for feeding, and yielded results that obscured differences between the potential feeding stimulants, we chose to conduct our assays with untreated Whatman 1, 55-mm diameter filter paper discs in the absence of a water source.

Three sets of laboratory bioassays were conducted following the standard protocol described above. In the first two sets, unsexed fourth instar nymphs and adult males and females of *M. sanguinipes* were used. Five vegetable oils were applied to filter paper discs: canola, flax, olive, rapeseed, and a 1:1 mixture of rapeseed and flax oils. In addition to the vegetable oils, as a positive control, we assessed an oil that is widely used as an adjuvant in agriculture sprays in the US: crop oil concentrate (United Agri Products brand), which is a paraffinic oil that contains a phosphate ester emulsifier. The third set of bioassays was conducted on fourth instar *M. sanguinipes* nymphs only. Ten vegetable oils were used: canola, corn, flax, olive, peanut, soybean, SoyStick (methylated soybean oil that is a chemically modified derivative of soybean oil often used as a non-ionic surfactant in herbicide sprays), sunflower, rapeseed, and rapeseed-flax 1:1 mix. As positive controls, we used both crop oil concentrate and mineral oil (Safeway brand, U.S.P.). The latter oil was composed of alkanes and cyclic paraffins derived from the distillation of petroleum that makes a colorless and odorless light oil.

In addition to the aforementioned qualities of oils, French rapeseed oil was tested because it was a carrier for an insecticide—Adonis® (a.i. fipronil)—used worldwide for grasshopper and locust control (FAO 2004). Flax oil was also tested in cooperation with Rhône-Poulenc, who was seeking at the time a highly available and economical carrier for other formulations of the insecticide fipronil. Although flax oil is usually not consumed by humans, it is high in linolenic acid.

Statistical Analyses In all three sets of assays, a minimum of ten replicates for each oil were tested in randomized order. The resulting data were analyzed with Pearson's chi-squared linear-by-linear association using SPSS 14.0 statistical software.

Table 3 Phagostimulation assay protocol development trial using 4th instar *M. sanguinipes* grasshopper nymphs. Two-choice tests of vegetable oils with and without water sources in the test arenas

Type of Oil	Pre-treatment Conditions	N Tested	N with Feeding Damage	Percent with Feeding Damage
None	Water	50	12	24
	No water	50	2	4
Crop	Water	10	1	10
	No water	10	1	10
Rapeseed	Water	10	10	100
	No water	10	2	20
Flax	Water	10	8	80
	No water	10	6	60
Rapeseed-Flax	Water	10	8	80
	No water	10	5	50
Canola	Water	5	5	100
	No water	5	3	60
Olive	Water	5	5	100
	No water	5	5	100

Results

Fatty Acid Analyses Fatty acid analyses of the vegetable oils studied and their percent similarity to *M. sanguinipes* are summarized in Table 2. Flax oil was the most similar to the fatty acid profile of *M. sanguinipes* (85%), followed in order by rapeseed-flax mix (71%), soybean (65%), peanut and rapeseed (56%), canola (54%), corn (53%), sunflower (47%), and olive oils (39%; Table 2). Peanut oil was the only oil to have any measurable quantity of erucic acid, a 22:0 carbon fatty acid. Both canola and rapeseed oils had less than 0.01% erucic acid. The latter was surprising because rapeseed oil typically contains between 25 and 50% of erucic acid (White 2000). As such, the rapeseed oil that we used should be considered as a variety of canola. However, the French rapeseed oil was unbleached and not deodorized, unlike canola or other food-grade vegetable oils tested. Unique to olive oil was the presence of 2% palmitoleic acid (16:1). The stearic acid (18:0) content of flax oil was closest to levels found in *M. sanguinipes* (Table 2). All analyzed vegetable oils lacked myristic (14:0) acid, which made up 1% of the fatty acid profile of *M. sanguinipes*.

Two of the tested oils, flax and sunflower, had higher amounts of linolenic and linoleic acids combined (72% each) than did *M. sanguinipes* (63%; Table 2). The other oils had lower amounts of these oils; olive oil had the least (13%), followed by canola (26%), peanut and rapeseed (29%), rapeseed and flax mix (50%), soybean (58%), and corn (60%).

The highest proportion of oleic acid (80%) was found in olive oil, followed by canola (65%), rapeseed (63%), peanut (52%), rapeseed-flax mix (41%), and corn and soybean (25%; Table 2). Flax oil had the same oleic acid content as *M. sanguinipes* (19%). Only sunflower oil contained a lower proportion (15%) of this fatty acid than the grasshopper.

Bioassays 1 and 2 Results of the first two sets of assays are presented in Tables 4 and 5. The untreated (negative control) papers had no nymphal feeding and a low level of feeding by adult grasshoppers (2 positives out of 60). Relative to the oil-treated papers, there was significantly ($P<0.05$) less feeding on plain paper than expected. The crop oil concentrate (positive control) had 10% feeding by the nymphs and 20% feeding by the adults. The positive control had significantly ($P<0.05$) less feeding than all of the vegetable oils except rapeseed. Feeding between vegetable oils differed from expectations ($P<0.05$). Olive oil was the most phagostimulatory of the oils, being fed on 100% of the time, by both nymphs and adult grasshoppers. Canola and flax had 60% feeding by nymphs and 80% feeding with adults. The rapeseed-flax mix had 50% feeding by nymphs and 70% by adults. Rapeseed oil had a 20% feeding response with nymphs and 70% with adults. Among the vegetable oils, only rapeseed showed significant ($P<0.05$) differences between the feeding of adults and nymphs.

Table 4 Assay 1: phagostimulation trial using fourth instar nymphs of *Melanoplus sanguinipes*

Oil	N	Percent with Feeding Damage	Preference Rank
None	60	0	6
Crop	10	10	5
Rapeseed	10	20	4
Flax	10	60	2
Rapeseed+flax	10	50	3
Canola	10	60	2
Olive	10	100	1

Table 5 Assay 2: phagostimulation trial using adult (50% males, 50% females) *Melanoplus sanguinipes*

Oil	N	Percent with Feeding Damage	Preference Rank
None	60	3	5
Crop	10	20	4
Rapeseed	10	70	3
Flax	10	80	2
Rapeseed+flax	10	70	3
Canola	10	80	2
Olive	10	100	1

Bioassay 3 Results of the third set of bioassays are presented in Table 6. The differences among vegetable oil treatments and the negative (untreated papers) and positive (mineral oil and crop oil concentrate) controls were significant ($P<0.01$). The oils could be grouped into those that induced low level feeding (crop 10%, rapeseed 20%, methylated soybean oil 20%); moderate feeding (flax 60%, rapeseed-flax mix 50%, canola 60%, peanut 60%, soybean 60%, and corn 70%); and heavy feeding (sunflower 90% and olive 100%).

Vegetable Oil Ranking The rankings of all studied vegetable oils with regard to three "theoretical" parameters (% similarity to *M. sanguinipes*, linolenic+linoleic acid content, and oleic acid content), as well as the results of three sets of bioassays are summarized in Table 7. Based on these data, the mean rank of every oil was calculated separately for theoretical expectations and for practical application of bioassays, and the oils were categorized accordingly (Table 8). The lowest rank reflected the highest attraction and phagostimulatory properties of oils to grasshoppers. Theoretical expectations resulted in the following ascending rank order: flax, rapeseed and soybean, canola and peanut, corn, rapeseed-flax mix, and, finally, sunflower and olive. Among the oils tested in all three sets of bioassays, olive oil performed best, followed by flax and canola, rapeseed-flax mix and rapeseed. Sunflower, corn, peanut, soybean and methylated soybean oils were tested only in one set of bioassays and corn and sunflower performed well.

Table 6 Assay 3: phagostimulation trial using fourth instar nymphs of *Melanoplus sanguinipes*

Type of Oil	N	Percent with Feeding Damage	Preference Rank
None	120	8	8
Crop Oil Concentrate	10	10	7
Rapeseed	10	20	5
Flax	10	60	4
Rapeseed+Flax	10	50	5
Canola	10	60	4
Olive	10	100	1
Corn	10	70	3
Mineral	10	0	9
Methylated Soybean	10	20	6
Peanut	10	60	4
Soybean	10	60	4
Sunflower	10	90	2

Table 7 Ranking of tested oils according to theoretical expectations and results of bioassays

Rank	Percent Similarity to *M. sanguinipes*	Linoleic+ Linolenic Content	Oleic Content	Assay no. 1 Nymphs	Assay no. 2 Adults	Assay no. 3 Nymphs
1	Flax	Flax Sunflower	Olive	Olive	Olive	Olive
2	Rapeseed+flax	Corn	Canola	Canola Flax	Canola Flax	Sunflower
3	Soybean	Soybean	Rapeseed	Rapeseed +Flax	Rapeseed Rapeseed +Flax	Corn
4	Peanut Rapeseed	Rapeseed+flax	Peanut	Rapeseed		Canola Flax Peanut Soybean
5	Canola	Peanut Rapeseed	Rapeseed +Flax			Rapeseed +Flax
6	Corn	Canola	Corn Soybean			Rapeseed
7	Sunflower	Olive	Flax			
8	Olive		Sunflower			

Discussion

Our expectations regarding the phagostimulatory properties of vegetable oils were based on two assumptions. First, the oil should have the fatty acid profile similar to *M. sanguinipes*. Second, to enhance feeding, the oil should have a significant amount of the fatty acids "behaviorally active" for grasshoppers (linoleic+linolenic, and oleic), and that are known to induce the insect movement toward a food source (Bomar and Lockwood 1994a, b, c). However, we found that none of the studied oils ranked consistently across all parameters. For example, the fatty acid composition of flax oil was most similar to *M. sanguinipes* (i.e.,

Table 8 Ranking of the tested vegetable oils according to theoretical expectations and results of three laboratory bioassays

Parameter/Rank Oil	Theoretical Expectation				Bioassays		
	Percent Similarity	Linolenic+Linoleic Content	Oleic Content	Mean	No. 1	No. 2	No. 3
Canola	5	6	2	4.3	2	2	4
Corn	6	2	6	4.7	–	–	3
Flax	1	1	7	3.0	2	2	4
Olive	8	8	1	5.3	1	1	1
Peanut	4	5	4	4.3	–	–	4
Rapeseed	4	5	3	4.0	4	3	6
Rapeseed+Flax	2	4	5	3.7	3	3	5
Soybean	3	3	6	4.0	–	–	4
Sunflower	7	1	8	5.3	–	–	2
Methyl. soybean	–	–	–	–	–	–	7

had the highest percent similarity), and had the highest content of linolenic and linoleic acids; however, it had one of the lowest proportions of oleic acid. In contrast, olive oil ranked lowest according to percent similarity and linolenic+linoleic acid content, but had the highest oleic acid content. Summation of the three theoretical parameters produced rankings that placed flax oil with the greatest potential, and olive and sunflower having the least, with the other oils somewhere in between.

There are reasons to be skeptical regarding the "theoretical expectations" criteria. Percent similarity comparisons were made to published data on the fatty acid content of adult *M. sanguinipes* (Barlow 1964). It is not known how close these data were to the actual fatty acid content of the tested insects, among which there were both nymphs and adults. Fatty acid content of oilseeds can vary depending on many different factors: for example, in the case of sunflower oil, even the position of the seed in the seed head matters (White 2000). Most probably, the fatty acid profiles of grasshoppers also vary depending on developmental stage, sex, source of food, etc. Hence, it is not surprising that our bioassay results did not agree fully with theoretical expectations. Despite its lowest "theoretical" rank, olive oil performed consistently and ranked as the best stimulant in all three sets of assays. It was followed by canola and flax, rapeseed-flax mix, and rapeseed oils. The five remaining oils were tested only once and, therefore, do not allow definitive conclusions to be drawn. Among them, sunflower and corn oil appeared to outperform peanut, soybean, and methylated soybean oils.

Laboratory bioassays demonstrate that addition of a standard aliquot of different vegetable oils result in varying degrees of grasshopper feeding on an otherwise neutral substrate. Addition of olive oil stimulated the greatest feeding in all three sets of assays, regardless of age of the tested insects. Furthermore, addition of canola or flax oils markedly enhanced grasshopper feeding. These three oils could be grouped together as best performing grasshopper stimulants. The second group included rapeseed-flax mix and rapeseed oils. Their performance was not as consistent, especially with regard to nymphal feeding. The third group consisted of soybean, corn, peanut, and sunflower oils. Theoretical expectations regarding these oils varied wildly, and results of a single bioassay should be cautiously interpreted as being negative.

Vegetable oils were shown to function as grasshopper attractants (Bomar and Lockwood 1994a, b, c). This quality makes the oils of particular interest when the insecticide to control a grasshopper infestation acts by ingestion (e.g., an insect growth regulator) and is applied with intervening untreated swaths. Such a management option is known as Reduced Agent and Area Treatment (RAAT; Lockwood and Schell 1997; USDA 2002). Since the mid-1990s, RAAT has been widely implemented on extensive areas in more than ten western states in the USA and in Eurasia (Lockwood et al. 2001; Latchininsky 2006). The ideal insecticide carrier would be attractive enough to move the target insect rapidly over tens of meters (the typical width of untreated swaths in RAAT programs) to the source and then phagostimulatory enough to prompt the insect to eat the treated substrate. This latter quality would both enhance the target efficacy of stomach poisons and reduce the impact on non-target arthropod fauna, compared to contact type insecticides. A vegetable oil that is both a kinetic and gustatory stimulant could be an ideal carrier for biological insecticides such as spores of the fungi *Beauveria bassiana* or *Metarhizium anisopliae*. Such a formulation would increase infection rates, as acridids seek out and eat the treated vegetation. Although fungal pathogens act by contact, the infection frequently occurs when spores enter the buccal cavity of grasshoppers along with food (Bidochka et al. 1997). Vegetable oils also may provide some UV protection to the fungal spores (S. Jaronski, *pers. comm.*). If so, this would also reduce the amount of the biological agent needed for control (Chernysh 2004).

Our laboratory bioassays are the first step in the search for an efficient, economical, and available carrier or adjuvant for insecticides used in grasshopper control. To validate our findings and generalize them towards other acridid pest species, other candidate oils need to be evaluated on operational-size plots under field conditions as has already been done with canola oil (Lockwood et al. 2001).

Acknowledgement The authors are indebted to Dr. Kirk A. VanDyke for invaluable assistance with experimental work and comments on the early version of the manuscript. We thank the anonymous reviewers for their comments and suggestions. The research project was funded in part through University of Wyoming, College of Agriculture mini-IPM grant and Wyoming Department of Agriculture WYAG49653 grant.

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Introduction

The transition of desert grasslands to shrub-dominated states is detrimental in areas where lands are valued for forage production for livestock. This transition, often accompanied by degraded conditions (e.g., soil erosion, decreased biodiversity, altered spatial complexity), is typically termed desertification (Pickup 1988; Peters et al. 2004) and has complex causes and effects (Reynolds and Stafford Smith 2002; Bestelmeyer 2005). *Flourensia cernua* DC (tarbush) is one shrub that is increasing in dominance at the expense of desert grasslands in the northern Chihuahuan Desert (Buffington and Herbel 1965). Dominance of shrubs in areas grazed by livestock is typically related to their palatability (Leopold 1924). Tarbush consumption by sheep and goats is negatively related to leaf surface concentration of individual terpenes and epicuticular wax (Estell et al. 1994a, b, 1998a). Herbivory/pruning of woody plants during dormancy can cause a reversion to juvenile growth form and increase the proportion of the plant that is current year growth, both of which are typically more heavily chemically defended (Bryant et al. 1991). Swihart and Bryant (2001) compiled 128 studies that examined a wide variety of herbivores and woody plants and found mature plants typically were preferred over juvenile plants. Mature woody plants have been reported to contain less camphor (white spruce; Sinclair et al. 1988), mono- and diterpenes (red cedar; Vourc'h et al. 2002), and total phenolics (13 African tree species; Rooke et al. 2004) than immature plants. Pisani et al. (2001) fed old and new growth from two *Prosopis* species (new growth was from plants that were topped 10 months earlier) and found that goats preferred old growth vs. new growth of *P. caldenia*, and that this difference was not explained by concentration of total phenolics or condensed tannins. No difference in preference for old or new growth was observed for the lesser preferred *P. flexuosa* in their study. Gowda (1997) observed no difference in concentration of total phenolics or condensed tannins in *Acacia tortilis* that were pruned (removal of outer 20 cm of all twigs) vs. intact plants, although physical defense (spine production) increased with pruning. Although several simulated browsing studies exist, few studies have examined effects of complete mechanical biomass removal on secondary chemical content in regrowth of woody species. Rea and Gillingham (2001) measured regrowth of Scouler's willow (*Salix scouleriana*) at different times after mechanical brushing (biomass removal 10–15 cm aboveground) and found tannin content was unaffected or lower than in intact plants. Our objective was to compare chemical profiles of the leaf surface of intact tarbush and regrowth. Our hypothesis was that regrowth would invest resources into defense compounds and contain greater terpene concentrations than intact plants.

Methods and Materials

Study Site The study was conducted on the Jornada Experimental Range in south-central New Mexico, USA. Soils on the site are deep, well-drained, Doña Ana-Reagan association and vary from sandy loam to loam (SCS 1980). The site is gently undulating with 1 to 5% slope. Long-term (1915 to present) mean annual and growing season (July–September) precipitation for the area is 247 and 131 mm, respectively. Long-term mean monthly temperatures for the coldest (January) and warmest (July) months are 6 and 26°C, respectively. Dominant vegetation on the study site is *Scleropogon brevifolius* Phil (burrograss) and tarbush (a deciduous, root-sprouting shrub). Study plants were located in a 7.5-ha paddock within a dense stand of tarbush from which livestock had been excluded for 5 yr before the study.

Sampling Protocol Ninety tarbush plants in the paddock were selected randomly and labeled at the base with an aluminum tag. All above-ground biomass (10 cm above soil surface) was removed at one time with lopping shears from 45 randomly selected plants during winter dormancy and removed from the site. Leaves were collected the following August during active growth from both regrowth and intact controls. Approximately 75 leaves (including petiole) were collected from a minimum of five leaders of current year's growth from each plant. Leaves were sampled from the middle one-third of leaders with forceps, placed on dry ice, and subsequently stored at −20°C.

Laboratory Analyses Five leaves from each sample were extracted in duplicate for 5 min at room temperature in 5 ml of 100% ethanol containing 5 ng/μl of 2-carene (internal standard) with occasional shaking, followed by filtration through a fiberglass (Fisherbrand G8) filter and stored at 4°C. Dry matter was measured in duplicate for each sample (10 leaves per duplicate) at 100°C for 24 hr. Leaf surface volatiles were analyzed by gas chromatography coupled to mass spectrometry, using a Finnigan ion trap mass spectrometer (EI, 70 eV) in conjunction with a Varian model 3400 gas chromatograph equipped with a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness 0.25 μm) with helium as carrier gas (1 ml/min), 1 μl injection size, and a programmed (injector temperature 220°C, transfer line temperature 240°C, initial column temperature 60°C, final column temperature 240°C, 3°C/min) temperature run (Adams 1995; Tellez et al. 1997). Identification of volatile compounds was performed by a comparison of mass spectra with authentic compounds when available or literature data (Adams 1995), and by a comparison of their relative retention times with those of authentic compounds or by comparison of their retention indices with those in the literature (Adams 1995). Concentrations of individual compounds were estimated by using the internal standard, and total volatile concentration was estimated from the cumulative concentrations of all compounds within a treatment.

Statistical Analysis Data were transformed to natural logarithms and subjected to univariate analysis of variance to compare mean concentration between resprouts and canopy for each compound and stepwise discriminate analysis to determine a subset of compounds that could discriminate between resprouts and canopy (SAS/STAT® 2002–2003).

Results

Of the 87 compounds present on tarbush leaves, 35 were greater in intact canopy samples and 16 were greater in regrowth samples based on univariate analysis ($P<0.05$; Table 1). The largest components (>100 μg/g of dry matter) were flourensadiol, unknown #1, artemisia alcohol, borneol, β-eudesmol, and unknown #4 for intact tarbush, and flourensadiol, unknown #7, unknown #6, artemisia alcohol, unknown #5, and β-eudesmol for tarbush resprouts (Table 1). Mean concentration of total volatiles on canopy leaves tended to be less ($P=0.062$) than that of regrowth (3642 vs. 4684 μg/g DM).

In the discriminate analysis, nine compounds (α-muurolene, iso-borneol, unknown #6, *p*-cymen-8-ol, unknown #7, sabinene, β-caryophyllene, δ-cadinene, and α-copaene) explained nearly 95% of the variation between canopy and regrowth samples, and α-muurolene alone explained almost 65% of the variation (Table 2). This subgroup was composed primarily of compounds in low concentrations (<20 μg/g of dry matter) with the exception of unknowns #6 and #7.

Table 1 Concentration and univariate analysis of volatile leaf surface chemicals on intact (control) and resprouted tarbush[a]

	Intact	Resprout	*P*
Total volatiles[b]	3643±358	4684±421	0.062
Santolina triene[c]	4.20±0.53	1.36±0.14	0.001
Tricyclene	13.34±1.61	3.04±0.50	0.001
α-thujene[c]	6.40±0.90	3.20±0.52	0.001
α-pinene	22.64±3.64	25.85±3.37	0.339
Camphene	80.29±11.27	81.96±12.02	0.607
Sabinene	3.42±0.83	6.17±1.18	0.016
β-pinene	13.24±2.27	17.78±3.12	0.202
Myrcene	20.70±4.91	21.19±5.55	0.468
Mesitylene[c]	3.53±0.57	2.57±0.49	0.064
Yomogi alcohol[c]	91.51±10.03	14.43±3.24	0.001
δ-3-carene	19.88±3.55	18.51±4.85	0.331
α-terpinene	3.04±0.40	1.22±0.17	0.001
p-cymene	9.57±2.03	7.81±0.95	0.868
Limonene	23.82±4.73	25.05±6.17	0.756
1,8-cineole	28.59±8.01	29.33±5.17	0.659
(*Z*)-β-ocimene	2.29±5.16	0.60±0.07	0.001
(*E*)-β-ocimene[c]	1.45±0.25	0.76±0.22	0.001
Trans-decahydronaphthalene[c]	2.10±0.39	0.49±0.05	0.001
γ-terpinene + artemisia ketone[c]	6.38±1.02	2.49±0.35	0.001
Cis-sabinene hydrate	10.56±2.49	13.23±2.46	0.408
Artemisia alcohol[c]	191.48±29.37	142.64±24.99	0.046
Terpinolene	2.49±0.53	1.23±0.22	0.001
Trans-sabinene hydrate	7.08±1.48	8.04±1.10	0.185
Cis-*p*-menth-2-en-1-ol[c]	10.25±1.22	3.61±0.59	0.001
α-campholenal[c]	3.32±0.55	1.13±0.40	0.001
Trans-pinocarveol[c]	5.99±0.96	1.67±0.47	0.001
Camphor + *trans*-verbenol[c]	8.31±1.20	2.55±0.41	0.001
Isoborneol[c]	3.03±0.27	0.42±0.06	0.001
Cis-chrysanthenol[c] + pinocarvone[c]	59.70±14.64	37.99±8.33	0.114
Borneol	168.94±25.48	74.26±15.82	0.001
Terpin-4-ol	11.27±1.51	3.44±0.77	0.001
m-cymen-8-ol[c]	2.10±0.20	0.38±0.06	0.001
p-cymen-8-ol[c]	3.82±0.44	0.52±0.08	0.001
α-terpineol	4.61±0.59	1.10±0.29	0.001
Myrtenal[c]	1.55±0.30	0.47±0.08	0.001
Myrtenol[c]	1.97±0.27	0.19±0.02	0.001
Cis-chrysanthenyl acetate[c]	3.85±0.55	1.07±0.19	0.001
Bornyl acetate[c]	3.94±0.51	2.30±0.36	0.009
Carvacrol[c]	5.38±0.60	0.80±0.12	0.001
α-cubebene[c]	2.41±0.32	2.66±0.44	0.855
Eugenol	2.51±0.39	0.38±0.07	0.001
Cyclosativene[c]	3.83±0.54	2.52±0.28	0.201
α-copaene	4.62±0.58	7.87±1.42	0.265
β-bourbonene[c]	8.54±0.62	7.19±0.76	0.072
β-cubebene[c]	6.72±0.60	4.97±0.60	0.034
(*Z*)-jasmone	23.33±2.80	23.64±3.15	0.544
Methyl eugenol	1.90±0.31	0.28±0.06	0.001
β-caryophyllene	17.65±1.50	11.72±1.24	0.001

Table 1 (continued)

	Intact	Resprout	*P*
α-humulene	11.84±1.23	5.91±0.69	0.001
Allo-aromadendrene[c]	2.29±0.37	3.09±0.53	0.511
Drima-7,9(11)-diene[c]	9.10±1.35	5.90±0.52	0.967
γ-muurolene[c]	3.16±0.66	4.03±0.45	0.007
Germacrene D[c]	27.82±3.77	19.20±2.61	0.167
β-selinene[c]	8.39±1.58	12.43±1.16	0.001
Epi-cubebol[c]	4.74±1.30	22.71±4.48	0.001
Bicyclogermacrene[c]	1.21±0.27	1.33±1.00	0.007
α-muurolene[c]	1.62±0.46	9.48±0.82	0.001
γ-cadinene[c]	10.86±1.77	31.47±6.94	0.012
Cis-calamenene[c]	1.65±0.37	6.13±0.95	0.001
δ-cadinene[c]	2.54±0.75	9.47±1.41	0.001
Cadina-1,4-diene[c]	6.78±1.33	3.46±1.83	0.116
Elemol[c]	9.73±1.81	7.67±1.60	0.263
Ledol[c]	48.65±9.47	37.49±4.31	0.852
Germacrene D-4-ol[c]	15.35±2.01	15.19±1.39	0.101
Spathulenol[c]	18.63±1.24	9.30±1.10	0.001
Caryophyllene oxide	29.99±3.99	27.22±4.58	0.423
Unknown#1	325.93±96.14	119.12±38.15	0.003
Unknown#2	38.79±3.77	30.4±3.47	0.055
β-oplopenone[c]	5.39±0.96	6.77±0.89	0.022
1-epi-cubenol[c]	23.42±1.79	35.40±2.85	0.002
Epi-α-muurolol[c]	6.30±1.16	3.54±0.59	0.094
(*Z*)-methyl jasmonate[c]	9.44±3.92	35.06±10.15	0.346
β-eudesmol[c]	140.61±9.88	118.35±11.10	0.080
Selin-11-en-4-α-ol[c]	76.33±9.82	28.83±3.95	0.001
Unknown#3	34.17±6.45	26.66±6.28	0.229
Bulnesol[c]	3.71±0.39	4.21±0.57	0.562
(*Z*)-methyl epi-jasmonate[c]	14.09±1.38	10.39±1.06	0.086
α-bisabolol[c]	8.24±1.93	40.39±6.35	0.001
Oplopanone[c]	3.30±0.49	6.73±0.78	0.001
Unknown#4	116.03±3.35	69.56±21.89	0.147
β-acoradienol[c]	21.28±1.44	8.75±1.01	0.001
Nootkatone[c]	7.39±1.04	12.41±1.76	0.003
Cryptomeridiol[c]	67.45±9.04	86.80±12.15	0.207
Flourensiadiol	1505.47±186.26	2238.83±294.24	0.786
Unknown#5	21.54±4.18	62.91±13.97	0.150
Unknown#6	29.32±13.62	197.91±18.58	0.001
Unknown#7	32.81±10.68	687.67±108.77	0.001

[a] Compounds were identified using kovats indices and mass spectral libraries; concentrations (± SEM; μg/g DM) were estimated from relative proportions of internal standard (2-carene); *N*=44 and 45 for regrowth and intact plants, respectively. Statistical analyses were conducted on natural logarithms of concentration means.

[b] Total volatiles are the cumulative estimated concentration of all compounds within a treatment.

[c] Tentatively identified based on Adams (1995)

Table 2 Stepwise discriminate analysis of volatile chemicals in intact (control) and resprouted tarbush

Order entered	Chemical	R^2
1	α-muurolene	0.645
2	Isoborneol	0.818
3	Unknown#6	0.874
4	*p*-cymen-8-ol	0.901
5	Unknown# 7	0.913
6	Sabinene	0.924
7	β-caryophyllene	0.933
8	δ-cadinene	0.942
9	α-copaene	0.948

Discussion

Concentration of carbon-based secondary metabolites in woody shrub species are well documented to be highly variable and affected by a number of factors, including plant species, class of compound, leaf age, plant age, season of year, induced responses from mechanical damage or herbivory, as well as a host of other environmental factors such as soil nutrients and light intensity (Cedarleaf et al. 1983; Bryant et al. 1991; Gershenzon and Croteau 1991; Powell and Raffa 1999; Massei et al. 2000; Ward and Young 2002). Pruning or herbivory during dormancy may cause woody plants to revert to juvenile growth stage and increase the proportion of current years growth, both of which are typically more chemically defended (Bryant et al. 1991). A number of theories have been forwarded regarding the allocation of plant resources to secondary metabolites, but a common theme is that tradeoffs exist between growth and defense (Herms and Mattson 1992). Young tissue is typically highly defended in slow-growing woody plants in low resource environments that rely predominately on constitutive carbon-based defenses (Bryant et al. 1991). Because regrowth from the crown and roots of shrubs represents a critical tissue that is paramount to survival of the plant, our hypothesis was that it would contain higher concentrations of secondary metabolites. However, our anecdotal observations suggest an alternative hypothesis because tarbush leaves from plants previously cut near the soil surface (roadgrader blading, etc.) are larger and more succulent in appearance, lighter in color, and less resinous and fragrant than leaves from uncut plants (visual observations only), suggesting they might contain lower concentrations of secondary compounds on the leaf surface. Increased palatability of regrowth after browsing has been reported for woody species in some cases (e.g., Danell et al. 1985; du Toit et al. 1990). Tarbush is a deciduous root sprouter, with new growth for both intact plants and regrowth occurring after summer rains. Both tissues were sampled on the same day; thus, leaf age and season were the same for this study, and any differences in leaf chemistry should be caused by the biomass removal treatment. Although plant age could not be specifically addressed, the 90 plants used in this study were fairly uniform in size and shape, and were assumed to be approximately the same age.

Because secondary metabolite concentration is closely related to consumption of shrubs by livestock and wildlife, the amount of defensive chemicals has implications for herbivory. Three of the first nine compounds to enter the discriminant analysis (sabinene, β-caryophyllene, and α-copaene) were unrelated to intake by lambs at concentrations found in tarbush (Estell et al. 1998b, 2000, 2005). Furthermore, none of the compounds tested (N=25) that did affect intake (α-pinene, camphene, caryophyllene oxide, and camphor) at

concentrations present in tarbush (Estell et al. 1998b, 2002) differed for regrowth with the univariate analysis (except camphor, which was inseparable from *trans*-verbenol in the present study). However, the total amount of volatile chemicals (cumulative estimated concentration of all volatiles within treatment) present on the leaf surface tended to differ (P=0.062) between intact and resprouted tarbush (Table 1). Intake of tarbush and other shrubs by ruminants has been related to total volatile concentration (Schwartz et al. 1980; Estell et al. 1994b; Riddle et al. 1996); thus, resprouts might be less palatable for livestock. However, it cannot be ruled out that other individual compounds that differed between intact and regrowth plants could affect palatability.

A number of studies have examined mechanical shrub removal in the context of shrub control and nutritional value (e.g., Kituku et al. 1992; Reynolds et al. 1992), but few report effects on secondary metabolite concentrations. Pisani et al. (2001) and Rea and Gillingham (2001) both reported lower concentrations of phenolics and/or tannins in regrowth of woody shrubs. This response is not completely surprising as these classes of compounds frequently increase in conjunction with structural components associated with maturity. Tannin content of blackbrush acacia regrowth 34 weeks after burning was greater than unburned controls, but honey mesquite and spiny hackberry regrowth tannin content did not differ from controls (Schindler et al. 2004). We are not aware of any studies that have examined terpenes in response to above-ground biomass removal for woody plants. Whereas mowing agronomic crops and pastures is well documented to reduce maturity and improve forage quality, less is known about the effects of altered growth stage in woody species. Our goal was to examine chemical changes associated with altering the vegetative state of tarbush by clipping intact plants and generating resprouts. Lower cumulative concentration of volatile compounds in intact plants than regrowth suggests repsrouts might be less vulnerable to herbivory than intact tarbush. From a practical standpoint, there would appear to be no advantage to altering growth stage as a means to improve tarbush consumption and reduce competition with grasses, although animal studies are needed for confirmation. Our findings support the general observations that young plants are more defended than older plants, in contrast to anecdotal observations mentioned above. Mowing tarbush and grazing the regrowth may not be an appropriate mechanism to promote grasses in tarbush-dominated desertified areas.

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despite defensive chemistry being a key plant fitness trait. Its expression across life stages is important in understanding the lifetime evolutionary selection pressures on a plant (Crawley 1997). Lack of a consistent pattern in chemical defense across different life stages may be a response to multiple herbivores. Alternatively, consistent expression of defense across multiple stages may arise if plants suffer attack from a single herbivore across multiple stages or if they utilize a similar suite of defenses to protect themselves against multiple herbivores (Leimu and Koricheva 2006). The investigation of variation in chemical defense within a genetic framework is crucial to interpreting observed variation in an evolutionary context. For example, Rehill et al. (2006) demonstrate variation in chemistry among developmental zones and the genetic basis of variation in developmental trajectories between parental and hybrid cottonwoods. They discuss their results in light of possible competing evolutionary pressures. Such studies are relatively sparse (Barton 2007), particularly in plant/mammal systems. This paper investigated the stability of genetic-based defensive chemistry to mammalian herbivores across life stages of a eucalypt species.

Phenotypic variation in defensive chemistry across life stages may be particularly marked in heteroblastic plant species, of which the species-rich tree genus *Eucalyptus* has many examples (Jordan et al. 1999). Eucalypts are prone to attack by a suite of vertebrate and invertebrate, generalist and specialist herbivores on different foliage types (juvenile and adult; Majer et al. 1997; Hume 1999). However, as in many plant species, the patterns of genetic-based defense across these stages are largely unexplored. Many heteroblastic eucalypts revert from the adult leaf phase to the juvenile phase when coppicing occurs after the main stem bearing the adult leaf type is damaged (Whittock et al. 2003). Coppicing involves re-sprouting from dormant basal buds at the base of the trunk and allows plants to recover from significant foliage loss often due to fire, wind, or herbivory (Vesk and Westoby 2004).

Eucalyptus globulus is a dominant tree in native forests in south-eastern Australia, and is also a major plantation species in temperate regions of the world (Dutkowski and Potts 1999). It exhibits heteroblasty during ontogeny (Jordan et al. 1999), and trees revert to the juvenile leaf phase when coppiced. In Australia, *E. globulus* foliage is consumed by invertebrates and several mammalian marsupial herbivores. Juvenile foliage is accessible to both terrestrial and arboreal marsupials, while adult foliage (present in a taller tree) is only accessible to arboreal marsupials. Juvenile *E. globulus* coppice foliage exhibits significant genetic-based variation in browsing resistance to a terrestrial macropod (*Thylogale billardierii*) and an arboreal possum (*Trichosurus vulpecula*; O'Reilly-Wapstra et al. 2002). There is also a genetic basis to the defensive compounds that confer resistance (O'Reilly-Wapstra et al. 2004). This suggests that mammalian herbivores have played a role in the evolution of population differences in defensive chemicals within this species. However, a key question is whether the same genetic-based differences in defensive chemistry of juvenile coppice foliage are evident across heteroblastic phases (in the adult foliage). Variation or stability in patterns of genetic-based defense across life stages may reflect the potential selective pressures imposed by associated herbivores at different times. In this work, we utilized *Eucalyptus globulus* trees of known genetic pedigree growing in a common environment trial in two different foliage phases (adult and coppice) to address the question of stability of defensive chemistry.

Materials and Methods

Foliage Collection Foliage was collected from a progeny trial situated at West Ridgley (41° 13′S, 145°80′E) in Tasmania, Australia. The field trial was established with open-pollinated

seed collected from 421 trees (families) from 49 localities throughout the natural distribution of *E. globulus* (see Potts and Jordan 1994 and Dutkowski and Potts 1999 for details). These localities were subsequently grouped into a spatial hierarchy of 13 geographic races and 20 subraces by Dutkowski and Potts (1999) based on spatial disjunctions and patterns of quantitative genetic differentiation observed in field trials. These races have subsequently been grouped into three major lineages based on microsatellite markers (Steane et al. 2006). Eight races of *E. globulus* were selected for this study based on their broad geographic distribution (Dutkowski and Potts 1999) and their genetic (Steane et al. 2006) diversity. The trial was a resolvable incomplete block design with five replicates of 23 incomplete blocks, and each family was represented as a two-tree line plot in each replicate. Ten families were randomly selected from the subrace typical of each of these races, and two trees from each family were selected from different field replicates. In autumn 2004, when trees were 15 years old, the 160 selected trees were felled. Leaves of healthy adult foliage were collected across the apical branch of each tree. The two trees per family were pooled to make 80 samples of foliage for chemical analysis. In autumn 2005, 80 coppice samples (ten families from eight subraces) were collected from the coppicing stumps of the previous years' cut trees. In this case, due to variable coppicing, only one individual per family was sampled, and in the few cases where neither tree coppiced (12 out of the 80), a substitute tree from another family from the same subrace was sampled. While the foliage we collected was accessible to herbivores, browsing of plants was minimal, and there is no evidence in the literature of induced chemical responses in eucalypts.

Analysis of Foliar Chemistry We assayed seven constituents in both the adult and coppice foliage. Two formylated phloroglucinol compounds (FPCs; sideroxylonal A and macrocarpal G, the two dominant isomers of these compounds in *E. globulus*) were assayed by high performance liquid chromatography (HPLC) following Wallis and Foley (2005). These compounds have strong anti-feedant properties to mammalian herbivores (Lawler et al. 2000; O'Reilly-Wapstra et al. 2004; Moore and Foley 2005). By using a pure standard of sideroxylonal A, we expressed results for this compound as mg g DM^{-1} (milligrams per gram of dry matter) and results for macrocarpal G are expressed as mg g DM^{-1} equivalents of macrocarpal A (by using a macrocarpal A standard).

Total oils and 1,8-cineole were assayed by gas chromatography-mass spectrometry (GC-MS; O'Reilly-Wapstra et al. 2004). From using a pure 1,8-cineole standard, the concentration of this compound is expressed as mg g DM^{-1}, and total oil is expressed as equivalents of cineole (mg g DM^{-1}). Total phenolics and condensed tannins were assayed with the modified Prussian blue assay for total phenolics (Graham 1992), and the acid butanol assay for condensed tannins (Porter et al. 1986). Foliage for these assays was prepared and extracted following the method outlined in Hagerman (2002). From using a gallic acid standard, the concentration of total phenolics is expressed as mg g DM^{-1} equivalents of gallic acid and, from using a sorghum standard, the concentration of condensed tannins is expressed as mg g DM^{-1} equivalents of sorghum tannin. Nitrogen was determined on air-dried foliage with the sulfuric acid and hydrogen peroxide method described by Lowther (1980) and analyzed colorimetrically on a continuous flow injection analyzer (QuickChem 800; Lachat Instruments).

Statistical Analyses For each chemical constituent, a one factor general linear model (PROC GLM, SAS, version 9.1) was used first to test the differences between adult and juvenile coppice foliage, and second to test the difference among subraces for each foliage type. Due to assumptions of normality and homogeneity, sideroxylonal A and nitrogen were

log transformed, and total oils and 1,8-cineole were square root transformed. Results for subrace analysis for each variable were analyzed separately, with a Bonferroni adjustment to account for multiple tests. Pearsons correlation coefficient was used to determine the relationship between the subrace mean levels of sideroxylonal A and macrocarpal G in the adult and coppice foliage (PROC CORR, SAS).

Results

There were distinct quantitative and qualitative differences in chemical constituents between the adult and coppice foliage samples (Table 1). Coppice foliage had substantially higher levels of most defensive compounds with higher levels of total phenolics ($F_{1,157}$=56.1, P< 0.001), total oils (√ transformed; $F_{1,154}$=494.3, P<0.001), 1,8-cineole (√ transformed; $F_{1,154}$=579.1, P<0.001), and sideroxylonal A ($\log_{10}$ transformed; $F_{1,157}$=219.5, P<0.001) than adult foliage. Levels of macrocarpal G were similar between the two foliage types ($F_{1,157}$=3.8, P=0.061) while there were no condensed tannins detected in the coppice foliage. Nitrogen was higher in the coppice foliage compared to adult foliage ($\log_{10}$ transformed; $F_{1,157}$=923.7, P<0.001).

Table 1 Chemistry (mean±SE) for adult and coppice foliage

		Adult Foliage			Coppice Foliage			Pearson's Correlation	
Variable	Units	Mean (±SE)	F_{df}	P	Mean (±SE)	F_{df}	P	R	P
Sider A	mg g DM^{-1}	2.0 (0.11)	$6.93_{7,77}$	<0.001[a]	6.7 (0.26)	$8.75_{7,79}$	<0.001	0.93	<0.001
Mac G	mg g DM^{-1} Mac A equivalents	7.3 (0.24)	$15.34_{7,77}$	<0.001	6.6 (0.20)	$5.53_{7,79}$	<0.001	0.95	<0.001
Tannin	mg g DM^{-1} Sorghum equivalents	9.3 (0.53)	$11.77_{7,75}$	<0.001	0	N/A	N/A	N/A	N/A
1,8-Cineole	mg g DM^{-1}	3.3 (0.14)	$0.53_{7,74}$	0.808[b]	27.8 (1.30)	$2.75_{7,79}$	0.014	−0.51	0.195
Total oil	mg g DM^{-1} 1,8−Cineole equivalents	7.8 (0.31)	$1.10_{7,74}$	0.399[b]	55.3 (2.68)	$2.20_{7,79}$	0.045	−0.69	0.060
Phenolics	mg g DM^{-1} gallic acid equivalents	15.2 (1.2)	$2.56_{7,77}$	0.021	39.9 (1.24)	$0.57_{7,79}$	0.781	−0.40	0.323
Nitrogen	% DM	1.2 (0.01)	$2.41_{7,77}$	0.029	2.2 (0.04)	0.82	0.573	0.10	0.810

Results are presented of the general linear model analysis of genetic variation at the subrace level within each foliage type. Significance levels for adult foliage are p<0.007 and p<0.008 for coppice foliage (which excludes the tannins) after bonferroni adjustment for multiple tests within foliage types. The Pearson's correlation indicates the relationship between foliage types at the subrace level

Sider A, sideroxylonal A; *mac G*, macrocarpal G; *mac A*, macrocarpal A; *tannin*, condensed tannin; *phenolics*, total phenolics

[a] Log10 transformed

[b] Square root transformed

There were significant differences among the eight subraces in the two FPCs (sideroxylonal A and macrocarpal G) for both the adult and coppice foliage, and condensed tannins in adult foliage, indicating a genetic basis to variation in these defensive constituents (Table 1, Figs. 1 and 2). At the subrace level, there was a strong positive correlation in the two FPCs between adult and coppice foliage (r=0.93, P<0.01; r=0.95, P<0.01 for sideroxylonal A and macrocarpal G, respectively), indicating stability of the genetic differences in compounds across these two markedly different life stages (Table 1, Fig. 1). In contrast, the differences (P<0.001) among subraces in levels of condensed tannins in the adult foliage has no analogue in the coppice foliage. This differentiation in adult foliage was due to the Strzelecki and Eastern Otway subraces having significantly higher levels of condensed tannins than the other subraces studied (Fig. 2).

Discussion

As highlighted by Barton (2007), few studies (but see Rehill et al. 2006) have documented genetic variation in ontogenetic patterns of defensive chemistry. Our study shows clear

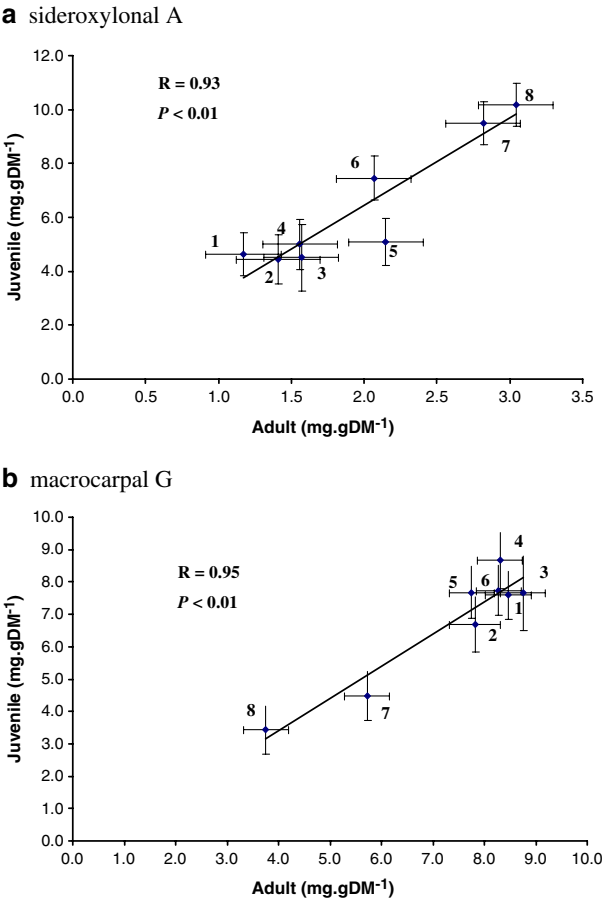


Fig. 1 Association between **a** sideroxylonal A (mg g DM^{-1}) and **b** macrocarpal G (mg g DM^{-1} equivalents of macrocarpal A) in adult and juvenile coppice foliage of *E. globulus*. Subraces are detailed in Dutkowski and Potts (1999) and correspond to: *1* King Island; *2* Southern Tasmania; *3* Eastern Otway Ranges; *4* Western Tasmania; *5* Western Otway Ranges; *6* South eastern Tasmania; *7* Flinders Island; *8* Strzelecki Ranges

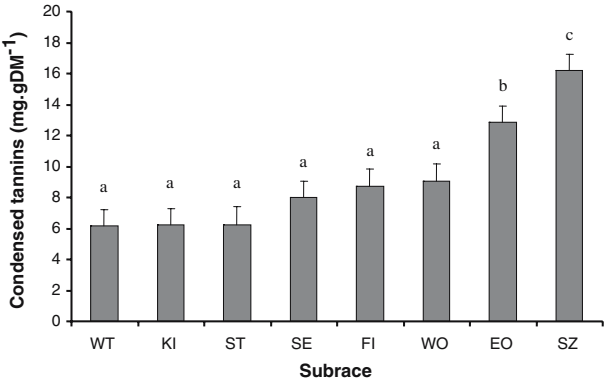


Fig. 2 Variation in condensed tannin levels [least square means (mg g DM^{-1} sorghum equivalents)±SE] of the adult foliage between the eight subraces. Letter that differ indicate significant differences (α=0.05) after Tukey-Kramer adjustment for multiple comparisons. *WT* Western Tasmania; *KI* King Island; *ST* Southern Tasmania; *SE* South eastern Tasmania; *FI* Flinders Island; *WO* Western Otway Ranges; *EO* Eastern Otway Ranges; *SZ* Strzelecki Ranges. a) sideroxylonal A, b) macrocarpal G

variation in the key ecologically important formylated phloroglucinol compounds (FPCs) in both the juvenile and adult foliage types of *E. globulus*. The patterns of variation of the two FPCs among genetically distinct subraces were tightly correlated across life stages, despite changes in the absolute levels of one compound. Our previous research (O'Reilly-Wapstra et al. 2004) and work by others (Moore and Foley 2005) have established the roles of these compounds in conferring resistance to marsupial herbivores that include *T. vulpecula*, the arboreal brushtail possum. The Strzelecki and Flinders Island subraces are differentiated from the other subraces in having higher levels of sideroxylonal A and lower levels of macrocarpal G for both adult and coppice foliage (Fig. 1a and b). A similar pattern has been observed in previous studies of coppice foliage (O'Reilly-Wapstra et al. 2002; 2004).

The FPC compounds are highly heritable (Andrew et al. 2006), and such strong genetic variation provides a platform on which selection can act. Previous research indicates browsing by *T. vulpecula* on young eucalypt plants results in lower reproductive output when the plants reach reproductive maturity, and also that feeding on juvenile coppice foliage provides direct positive selection on sideroxylonal A (O'Reilly-Wapstra et al., unpublished data). The correlated variation in FPCs at the subrace level across life phases may arise from two different scenarios. First, the patterns could be a consequence of adult and coppice defensive chemistry being under the control of the same genes, the differences in expression of which is little modified with life stage. In this scenario, selection through marsupial browsing at either life phase would elicit a correlated response in the other life phase. However, given that *T. vulpecula* feeds on both juvenile coppice (O'Reilly-Wapstra et al. 2004) and adult foliage (Wiggins et al. 2006), it is possible that with geographic variation in browsing pressure from this marsupial, independent selection could result in correlated patterns of genetic variation in chemicals across life history stages, even in the absence of common genetic control.

The strength of selection at different life stages of a plant will be determined in part by the relative fitness costs of having foliage browsed. Coppice re-growth is a response to foliage loss of the main stem, and relative costs of herbivory on the comparatively small biomass of this foliage would be far greater compared to herbivory on a large biomass of adult foliage in a reproductive, mature tree (Swihart and Bryant 2001). In our system,

coppice foliage is susceptible to browsing by two marsupial herbivores (*T. vulpecula* and *T. billardierii*, the terrestrial macropod; O'Reilly-Wapstra et al. 2002), and the levels of most plant secondary compounds (sideroxylonal, total oils, cineole, and total phenolics) were higher in coppice foliage than adult foliage. This supports the idea that allocation of chemical defenses in *E. globulus* is greater in foliage that potentially suffers more browsing pressure and has higher fitness consequences if browsed. This line of thought has been supported in numerous plant/mammalian systems where young foliage in early ontogenetic stages is generally well defended compared to older foliage (review Swihart and Bryant 2001).

There is considerable evidence for the role of tannins in herbivore defense (Harborne 1991 and references within). Tannins, however, also play other roles such as acting as antioxidants (Hagerman et al. 1998) and protecting plants against photo damage (Close and McArthur 2002). In this paper, we found no stability in the expression of condensed tannins between life stages of *E. globulus*. That is, the clear genetic variation observed in the adult foliage was not evident in the coppice foliage because of a lack of tannins in the juvenile phase. In support of this result, coppice foliage in a partner field trial (data not shown) does not contain detectable levels of condensed tannins. Coppice foliage from artificially browsed lignotubers on *E. globulus* seedlings also lacks condensed tannins (O'Reilly-Wapstra, unpublished data). These compounds, however, are present in juvenile seedling and adult foliage in *E. globulus* and other eucalypt species (Macaulay and Fox 1980; Gras et al. 2005), and the defensive function of tannins cannot be discounted (Marsh et al. 2003; O'Reilly-Wapstra et al. 2005). It is possible that the lack of a consistent pattern in condensed tannins across life stages in *E. globulus* may be a response to multiple herbivores (including both mammals and invertebrates) that consume the plant. Specific compounds may be effective against specific herbivores. However, if tannins play a role in defense to mammalian herbivores in this system, we would expect that one of the most vulnerable and highly accessible life stages (coppice) would contain tannins. In addition, our previous work in this system shows that the levels of condensed tannins are phenotypically plastic, particularly when compared to the strongly constitutively stable and highly defensive FPCs (O'Reilly-Wapstra et al. 2005). Such plasticity in tannin levels is evident in other systems (e.g., Aspen, see Osier and Lindroth 2001). These results together may lend support to alternative hypotheses for the role of tannins in the eucalypt system, such as protection against photo damage (Close and McArthur 2002). It is not until the multiple roles of condensed tannins and trade-offs with their production in eucalypts are more fully investigated that we will clearly understand their role in defense against mammalian herbivores.

Acknowledgement Funding was provided to J.O'R-W and B.P. by the Australian Research Council and the University of Tasmania Institutional Research Grant Scheme. We thank Gunns Ltd. and Kelsey Joyce for access to foliage, Robert Barbour, Hugh Fitzgerald, Simone Janney and Noel Davies for aspects of foliage collection and foliage chemical analysis, and an anonymous reviewer for comments on the manuscript.

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Keywords Allelochemicals · Aminophenoxazines · APO · *Bacillus mojavensis* · Benzoxazinoids · 2-benzoxazolinone · 2-amino-phenoxazin-3-one · BOA · Cyclic hydroxamic acids · DIMBOA · *Fusarium verticillioides* · Maize · MBOA · Microbial transformations · *Zea mays*

Introduction

Benzoxazinones and their decomposition products (benzoxazolinones and their methyl derivatives) are constitutive naturally occurring allelochemicals of wheat, corn, rye, other members of the Gramineae (Niemeyer 1988), and some non-Gramineae species (Pratt et al. 1995; Baumeler et al. 2000). These compounds serve as plant-defensive metabolites, providing protection from fungi, insects, and invertebrate pests, and they also are allelopathic. Because of their wide spectrum of biological activity, they offer promise for natural controls of pests. The main benzoxazinones produced by maize are 2,4-dihydroxy-7-mcthoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), and its dimethoxy derivative 2,4-dihydroxy-6,7-dimethoxy-1,4-benzoxazin-3(4*H*)-one (DIM_2BOA), with the former occurring in greater concentrations (Whitney and Mortimore 1959; Klun and Robinson 1969; Corcuera et al. 1978; Copaja et al. 1991; Hedin et al. 1993; Frey et al. 1997; Cambier et al. 2000). These benzoxazinones are present in maize mainly as glycosides, but upon injury from insects or pathogens they are degraded to their unstable and highly toxic agluconic forms, which spontaneously decompose to the corresponding benzoxazolinones, such as MBOA (6-methoxy-2-benzoxazolinone) and BOA (benzoxazolin-2-one) (Niemeyer 1988). Thus, MBOA and BOA are found in plant debris and soil as stable degradation products of DIMBOA and DIBOA, and are considered potentially useful allelochemicals (Fomsgaard et al. 2004).

Maize begins to synthesize and store DIMBOA and DIM_2-BOA as the glycosides once germination is initiated. Many insects, fungi, and bacteria are deterred or inhibited by these benzoxazinoids, resulting in increased plant resistance (Virtanen and Hietala 1955; Wahlroos and Virtanen 1958; Whitney and Mortimore 1959; Klun and Robinson 1969; Couture et al. 1971; Argandona et al. 1980; Givovich and Niemeyer 1991; Hartenstein and Sicker 1994; Koziel et al. 1996; Frey et al. 1997; Hansen 2006). The main fungal inhabitant of maize is the anamorph of *Gibberella moniliformis*, *Fusarium verticillioides* (=*Gibberella fujikuroi* mating population A; syn., *F. moniliforme*). This ascomycetous fungus is consistently found in association with maize throughout the world as a symptomless endophyte (Kommedahl and Windels 1981; Marasas et al. 1984; Bacon and Hinton 1996). The symptomless endophytic association with maize is apparently evolutionarily relevant, as it has been established that *F. verticillioides* and other endophytic fungi detoxify DIMBOA/MBOA and DIBOA/BOA by actively transforming them into the nontoxic components *N*-(2-hydroxy-4-methoxyphenyl) malonamic acid (HMPMA) and *N*-(2-hydroxyphenyl) malonamic acid (HPMA), respectively (Virtanen and Hietala 1955; Zikmundova et al. 2002; Glenn et al. 2003).

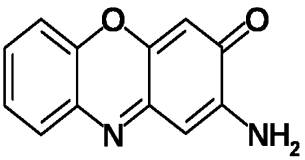
Fusarium verticillioides is a nonobligate pathogen; it is not host-specific and has been reported in association with many plant species in addition to maize. It is estimated that 11,665 plant species are potential hosts of *F. verticillioides* (Bacon et al. 1996), although this number may be confounded by historical confusion over current fungal and plant species delimitation and taxonomy. *F. verticillioides* has a long history of being associated with animal toxicosis, and only in this past decade has the etiologic agent been isolated and identified as the fumonisins (Marasas 1996). After this discovery, this class of mycotoxins was established as the cause of equine leukoencephalomalacia, porcine pulmonary edema, and experimental liver cancer in rats. The consumption of maize contaminated with this

fungus is associated with human esophageal cancer and fetal neural tube defects (Voss et al. 1989; Wang et al. 1991; Riley et al. 1993, 1994; Waes et al. 2005). Thus, control of this cosmopolitan fungus on maize is important.

The benzoxazinoids offer protection to the plant from pathogenic attack from non-detoxifying microbes. After the very broad definition of the International Allelopathy Society (Torres et al. 1996), they can be considered allelopathic. However, the ability of fungi to detoxify these or other allelochemicals may allow for an endophytic coexistence with maize, resulting in a competitive advantage for resistant fungi (Glenn et al. 2003). Prior studies established that several species of *Fusarium* are tolerant to the benzoxazinoids (Glenn et al. 2001), and that this tolerance in *F. verticillioides* is in fact caused by the presence of at least two genetic loci, FDB1 and FDB2. Detoxification of the benzoxazolinones requires both loci, and defect at either locus causes sensitivity to the compounds (Glenn et al. 2002). A new transformation product is produced from 2-aminophenol (AP). This product, 2-acetamidophenol (HPAA), was identified as a transformation compound produced by several genetic strains of *F. verticillioides* (Glenn et al. 2003). Several other fungi and bacteria are also known to detoxify this class of compounds (Friebe et al. 1996, 1998; Wieland et al. 1999; Vilich et al. 1999), although genetic mechanisms of detoxification, if any, have not been determined.

Tolerance to the benzoxazinoids by plant pathogens precludes these allelochemicals as control strategies for some pathogens. However, there are several microbial transformation products of BOA that are alleleopathic of which the aminophenoxazine 2-amino-3H-phenoxazin-3-one (APO) (Fig. 1) is stable in soils (Macias et al. 2005). The toxicities of APO compared to other transformation products differ by several orders of magnitude higher than any other benzoxazolinone metabolites (Atwal et al. 1992; Fritz and Braun 2006; Martyniuk et al. 2006).

In a study designed to exploit the natural chemical defenses of corn in combination with the patented endophytic bacterium, *Bacillus mojavensis*, as a biocontrol of *F. verticillioides*, a semiochemical interaction, indicated by the formation of an intense red diffusible pigment, was observed when the two are cultured together on media amended with BOA. The endophytic bacterium has been patented for control of disease on maize, primarily control of the fungus *F. verticillioides* and mycotoxin reduction (Bacon and Hinton 1999). However, the activity of the relatively stable benzoxazolinones on this nontarget endophytic bacterium is not known, and its biocontrol activities might be compromised if it is sensitive to the benzoxazolinones and their decomposition products. As these maize allelochemicals exist in high concentrations early during maize germination (Klun and Robinson 1969) and it is at this time that the biocontrol bacterium, *B. mojavensis*, infects corn seedlings (Bacon and Hinton 1996), the objectives of this research were: to determine the interactive nature of BOA and its final transformation product, APO, on the growth of *B. mojavensis*, and to



2-amino-3*H*-phenoxazin-3-one (APO)

Fig. 1 Structure of 2-amino-3*H*-phenoxazin-3-one (APO), a degradation product of 2-benzoxazolinone (BOA)

determine the *in vitro* antagonistic activity of the bacterium on the biocontrol target *F. verticillioides* cultured in the presence of BOA.

Methods and Materials

Allelochemicals Chemicals used for the transformation analysis were obtained from the following sources: BOA and 2-aminophenol from Aldrich Chemical Co., Milwaukee, WI, USA; MBOA from Lancaster Chemicals, Lancaster, Ca, USA; HPMA was purified and identified after transformation of BOA by RAC826 followed by TLC (Glenn et al. 2003). The identities of these compounds were based on prior analysis and chemical confirmation that used TLC and mass spectrometry procedures reported earlier (Glenn et al. 2003). APO was prepared and chemically characterized by previously described procedures (Macias et al. 2005).

Fungal and Bacterial Strains The strains of fungi used were those that either tested positive for detoxification of BOA or those with defective alleles at loci necessary for detoxification of BOA (Glenn et al. 2002). The *F. verticillioides* strains used consisted of MRC826 (genotype FDB1/FDB2), a wild type for fumonisin production and tolerance to BOA, strain AEG73-A4-1, a tolerant benzoxazolinone phenotype (genotype *Fdb1/Fdb2*) derived from a cross of the wild-type *F. verticillioides* RRC826 with the female fertile AEG 3-1-6 sensitive genotype, and AEG73-A4-2 (genotype *Fdb1/fdb2*), a sensitive benzoxazolinone phenotype derived from this same cross (Glenn et al. 2002). For long-term storage, conidia were frozen at −80°C in 15% glycerol. For routine culturing, spores were streaked onto potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) and incubated at 22°C in the dark. The bacterial strains used were *B. mojavensis* including the patented biocontrol strain RRC 101 (ATCC 55732), and its rifamycin-resistant mutant, RRC112 (Bacon and Hinton 2002). The bacteria were stored as silica gel cultures at −20°C, or 10% glycerol at −80°C, and working cultures were maintained on nutrient agar (Difco Laboratories, Detroit, MI, USA) at 4°C. Inoculum of each strain was prepared from overnight shake cultures grown in nutrient broth (Difco Laboratories, Detroit, MI, USA) at 30°C.

Bacterial–Fungal–BOA Interactions Experiments designed to determine bacterial antagonism to the fungus were assessed on nutrient agar. Antagonism experiments were performed by placing bacterial inoculum prepared from a 24-hr nutrient broth culture (10^4 CFU/ml) either down the center or 2 cm from the edge of each plate, and incubated 2–4 d at 30°C. Plates were then inoculated with a fungal inoculum (10^4 CFU/ml) from a 5- to 7-d-old fungal culture on either or both of the opposite edges of a Petri plate, which was incubated for 7 d at 25°C. The zone of inhibition between the bacterium and fungus was measured on all test medium following earlier procedures (Bacon and Hinton 2002). Radial growth measurements were made 7 and 14 d after inoculation in four directions from the edge of the inoculum to the advancing margin of the colony. Additional observations were made 15 d after inoculations to note any differences in growth responses. Each experiment was repeated at least twice with three replications for each treatment.

Microbial Transformation in Liquid Culture Flasks, 125 ml, containing nutrient broth (50 ml/flask) amended with BOA, 1.0 g l^{-1}, were inoculated with the bacterium prepared from 24-hr nutrient broth cultures (10^6 cell/ml), and incubated on a shaker, 100 rpm, for 24 hr at 25°C. The fungal inoculum (10^4 cfu/ml), which was prepared from 5- to 7-d-old PDA cultures, was added to the BOA-amended nutrient broth immediately after the bacterial

inoculum, placed on the shaker (150 rpm), and incubated for 5–7 d in the dark, and checked for bacterial–fungal interactions indicated by the presence of the red color of APO. Control flasks consisted of nutrient broth with and without BOA or with comparable amounts of ethanol and inoculated either with or without the bacterium or the fungus. Solvent extractions and analyses of liquid media were performed according to Yue et al. (1998). Each experiment was repeated at least twice with three replications for each treatment.

Tolerance of Bacillus mojavensis to BOA and APO Growth rate experiments were used to measure the toxicity of BOA or APO to *B. mojavensis*. Experiments were performed with an automated turbidometer, the Microbiology Bioscreen C Reader (Labsystems, Helsinki, Finland), which was conduced in 100-well sterile microplates. Each well contained 190 µl of nutrient broth, with or without BOA or APO, to which was added 10 µl of bacterial inoculum (10^3 CFU/ml) or fungal inoculum (10^6 CFU/ml). The cultures were incubated at 30°C with constant shaking, and the optical density, OD_{600}, was measured at 30-min intervals over the incubation period of 48–72 hr. The concentrations of BOA used were 1, 0.5, and 0.25 mg/ml. Fungal and bacterial cells from 2-wk-old BOA-amended cultures were removed by filtering through a sterile membrane (0.2 nm), and diluted 50% or 25% with nutrient broth. The dilutions were inoculated with either the fungus or the bacterium. Growth curves and several other plot parameters were generated and analyzed by the software package of the Bioscreen C Reader (Research Express, version 1.00). The specific rates of growth were expressed as a percent reduction over controls that contained no BOA. All experiments were performed in triplicate and were repeated independently. The reported specific rates of growth are averages from two or more experiments, and data were evaluated by analysis of variance and Student's *t* test analyzed by the Analysis ToolPak Microsoft Excel, version 2003 (Microsoft Corp.).

Chemical Transformation of BOA in the Presence of Bacillus mojavensis The interactions of BOA metabolism in the presence of the bacterium and fungi were determined by the thin layer chromatography (TLC) procedure described earlier (Glenn et al. 2001). All strains were grown on 1.0 mg/ml BOA for approximately 7 d, and an agar plug (8 mm diameter) was taken from both the advancing margin of the colony and from within the colony. The agar plugs were then transferred to a TLC sheet (Whatman; silica gel, UV_{254}, aluminum backing), which was heat activated at 50 °C for 10 min before application of the plugs. Plugs were placed on the sheets with the lower surface of the plugs in contact with the silica gel. The contents from plugs were allowed to absorb into the silica gel for approximately 10 sec. Three plugs from each location were placed on the same spot, with drying of the spot between each application with a heat gun, following the procedure of Glenn et al. (2001). Sheets were developed in a saturated chamber containing toluene, ethyl acetate, formic acid (50:40:25, *v*/*v*). Developed sheets were visualized and photographed under UV light by using digital imaging (Alpha Innotech), and R_f values were compared with standards of authentic compound.

The identity of APO resulting from biotransformation of BOA by *F. verticillioides* was indicated by its bright red color (Anzai et al. 1960; Gagliardo and Chilton 1992) and by comparing TLC R_f values with standards, as well as by comparing the absorption spectra with authentic APO after eluting it with methanol from the silica gel (Glenn et al. 2003; Fomsgaard et al. 2004). Further, the confirmation of APO as the decomposition product was based on established published procedures by comparison to a standard of the authentic compound (Macias et al. 2005; Fomsgaard 2006) by using absorption spectroscopy, and gas

chromatography-mass spectroscopy (GS-MS), described earlier (Glenn et al. 2001, 2003; Fomsgaard 2006).

Results and Discussion

The general inhibitory data and growth measurements as well as responses to the fungus by the bacterium are similar to the data described earlier (Bacon and Hinton 2002) and are pictorially shown in Fig. 2a. *B. mojavensis* produces a soluble inhibitory substance on nutrient agar that prevents the growth of *F. verticillioides* from making contact with the

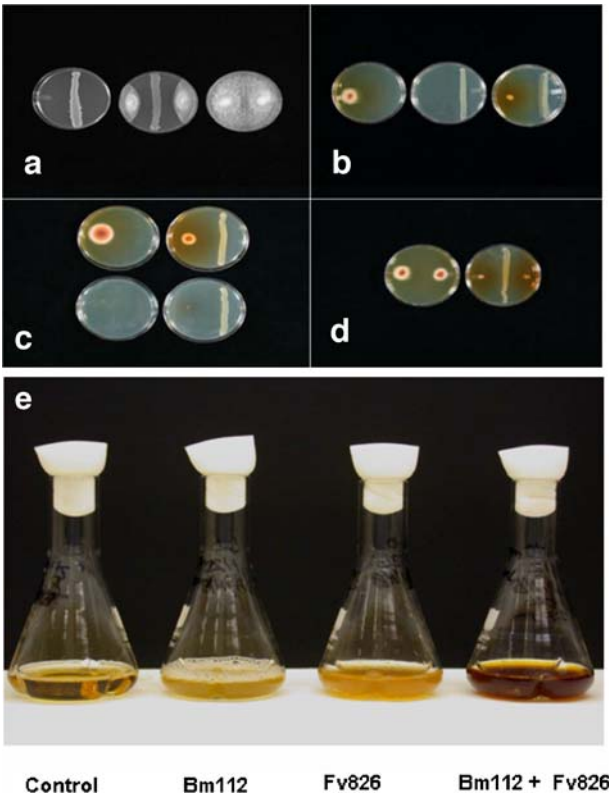


Fig. 2 Culture of *Bacillus mojavensis* RRC102 and *Fusarium verticillioides* MRC826 on nutrient agar with and without BOA. (**a**) Nutrient agar cultures showing zones of inhibition produced by the bacterial inhibitor after 14 d of culture, *center plate*, compared with growth of the fungus cultured alone, *far right plate*. (**b**) Culture of *Fusarium verticillioides* cultured with and without *B. mojavensis* on BOA-amended nutrient agar showing production of *red pigment* (APO), and on the *far right* is a colony of the severely inhibited fungus observed when co-cultured with the bacterium. (**c**). *Top row*, production of APO, the *red pigment*, by *F. verticillioides* (RRC826) with (0.5 cm) and without (1.6 cm) the bacterium *B. mojavensis* (RRC 112); *bottom row*, agar plates showing no growth with and without one of the benzoxazolinone-sensitive genetic strains of *F. verticillioides,* AEG-73-A4-2. (**d**) Production of APO (*red pigment*) in the presence of *F. verticillioides* RRC826 alone (*left*), and with the bacterium (*right*). (**e**) Liquid culture of *B. mojavensis* (*Bm112*) and *F. verticillioides* (*Fv826*) on nutrient broth amended with BOA (1.0 g l^{-1}) resulting in the production of APO during single and dual culture of the organisms (Bm112 and Fv826)

bacterial culture. This inhibitor has been partially characterized as one of the cyclic lipoprotein inhibitors characteristic of this genus of bacteria (Bacon et al. 2005). This bacterium has *in vitro* inhibitory activity to several known maize and other cereal pathogens (Bacon and Hinton 2002), including the ability to reduce the amount of the fumonisin mycotoxins produced by *F. verticillioides* in corn (Bacon et al. 2001). However, the interaction is complex as it was determined that *F. verticillioides* produced fusaric acid, 5-butylpicolinic acid, that is an antibiotic to *B. mojavensis* and other species (Bacon et al. 2006), interfering with the biocontrol potential of bacteria. The *in planta* accumulation of these allelochemicals, to which the *Fusarium* species have been shown to be tolerant (Glenn et al. 2001), is expected to complicate the use of endophytic bacteria for biocontrol work. However, the results of this study indicate that the major benzoxazolinone in maize, BOA, is not toxic to *B. mojavensis*, and that it is transformed in the presence of the bacterium and the fungus, resulting in a product that is further inhibitory to the fungus but not the bacterium. This is in contrast to what the fungus can do alone.

On BOA-amended medium, the inhibitory response observed on nutrient agar is intensified, and greatly restricts the growth of *F. verticillioides* compared to the inhibition observed on media without BOA (Fig. 2b). On BOA-amended medium and in the absence of the fungus, there is no production of the red pigment, even after an extended incubation period of several weeks (data not shown). During an extended incubation, the fungus degraded the BOA, producing radial growth similar to control, which was reported earlier (Glenn et al. 2001). However, on the BOA-amended medium and when both organisms are co-incubated, the red pigment is produced as early as the seventh day of incubation (Fig. 2b,c). Extraction of media, both solid and liquid, determined that this pigment formed in these cultures was APO, based on HPLC, absorption spectra, and biological activity of the compound on *F. verticillioides*. APO was isolated and chemically identified by Gagliardo and Chilton (1992) from nonsterile soil that had been supplemented with BOA. Sensitivity of *F. verticillioides* toward the inhibitory substance produced by *B. mojavensis* was enhanced greatly when cultured on the BOA-amended nutrient agar because of the production of APO. This severe inhibition was not observed on PDA amended with BOA (data not shown), as on PDA the bacterium does not produce the inhibitor. This suggests that the more restricted growth of the fungus is caused by activity of both the accumulation of APO and to the bacterial inhibitor. During an extended incubation period, the growth is still restricted when the fungus is cultured on BOA-amended media in the presence of the bacterium (Fig. 2d), and more severely restricted than when grown in the presence of the bacterium and in the absence of BOA (Fig. 2a).

An intense accumulation of APO, indicated by a large zone of deep red pigment, in the BOA-amended medium appears on media in the presence of the bacterium. The amount produced in the presence of the bacterium appears to be more, and the color is red as opposed to a yellow-red observed in culture without the bacterium (Fig. 2c,d). The inhibition observed by the bacterium (Fig. 2a) compared to that produced in the presence of BOA-amended nutrient agar is greater (Fig. 2b,d). The progeny of the two genetic crosses of *F. verticillioides* do not grow on the BOA-amended medium, and there are no accumulation patterns of the red pigments (Fig. 2c), indicating that fungal growth and activity are required for the production of transformation products from BOA. It is established that the transformation of BOA follows several steps that lead first to 2-aminophenol (AP), which is a microbial transformation product (Nair et al. 1990; Gagliardo and Chilton 1992; Glenn et al. 2002; Fomsgaard et al. 2004), and subsequently microbial transformations or natural oxidative processes lead to the formation of 10 amino-phenoxazinones (Fomsgaard et al. 2004). That *B. mojavensis* is required for the

accumulation of APO is indicated in those cultures that showed a greater intensity of pigment accumulation over cultures that did not contain both the bacteria and fungi, and absent in nutrient broth without the addition of BOA (Fig. 2e). Another maize pathogen, *Gaumannomyces graminis* var. *tritici*, transformed BOA to APO in liquid culture (Friebe et al. 1998), and APO is now known to occur in cultures of several root endophytic fungi of tobacco, including *F. sambucinum* (Zikmundova et al. 2002).

The red color observed on agar media amended with BOA is also produced in nutrient broth by the bacterium-and-fungus mixed culture (Fig. 2e). Most of this color is caused by APO, which is based on its absorption spectrum of extracted media, and R_f values of the red spots on TLC plates (Fig. 3). However, there were other minor products observed on the TLC plates, suggesting the possible presence of related aminophenoxazinones. It is evident that the bacterium does not produce APO when exposed to BOA (lane 10, Fig. 3), and that APO is produced only in the presence of the fungi (lanes 4–9). Further, these data also indicate that this bacterium is not capable of transforming BOA (lane 10, Fig. 3). HPMA, a major decomposition product of BOA, was also obtained in lanes 4–9, but in the culture of the bacterium, lane 10, there was no decomposition product, and only the original BOA remained with intensity similar to the standard, lane 1. However, the intensity of the BOA in lanes 4–9 decreased, indicating transformation by the fungus. Apparently, the tolerant cross, AEG73-A4-1, lane 9, transformed BOA similarly to the pattern identified in the wild-type strain resulting in AP, which is transformed or oxidized to APO and HPMA, and as discussed below to 2-acetaminophenol (HPAA) by strains defective at appropriate genetic loci.

APO was first described as an antifungal substance under the name of questiomycin A, a compound produced from *Actinomycetes*, *Pseudomonas*, and *Waksmania aerate* (Anzai et al. 1960; Gerber and Lechevalier 1964). Gagliardo and Chilton (1992) later reported that APO was more toxic to microorganisms than BOA. The toxicity spectrum for the aminophenoxazinones for those organisms tested is APO>HPMA>BOA (Fritz and Braun 2006). Our study, however, indicated that neither APO nor BOA was toxic to *B. mojavensis* (Fig. 4) at concentrations that occur naturally (Cambier et al. 2000). However, there was a significantly longer lag period at the 25-μg/ml concentration than obtained with the other concentrations (Fig. 4). APO was toxic to *F. verticillioides* when cultured on the BOA-amended agar-based media used here, which suggests that the fungus was not able to

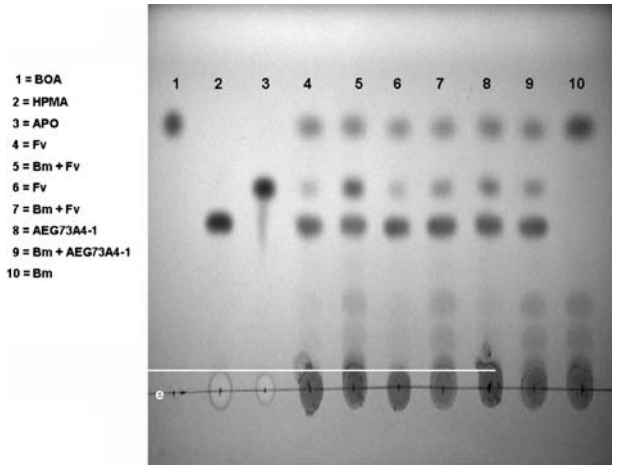


Fig. 3 TLC profiles of BOA and related metabolites obtained from agar plugs placed on silica gel plate showing the transformation products obtained from a 7-d-old culture of *F. verticillioides* MRC 826 (FV) and one of the genetic strains, AEG73-A4-1, cultured alone and in combination with *B. mojavensis* RRC112 (*Bm*); BOA, benzoxazolin-2-one; HPMA, *N*-(2-hydroxyphenyl) malonamic acid; APO, 2-amino-3H-phenoxazin-3-one. The silica gel plates were prepared following the procedure of Glenn et al. (2001)

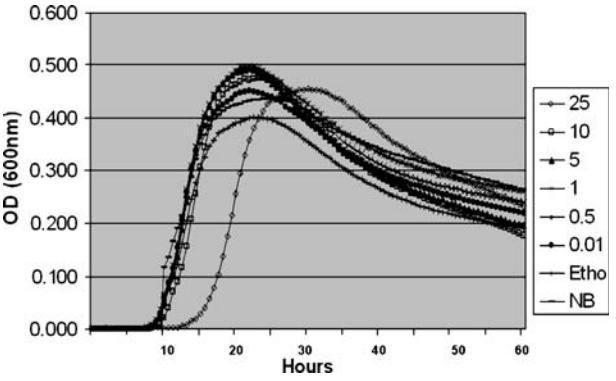


Fig. 4 Effects of APO on the specific rates of growth of *Bacillus mojavensis* RRC101 cultured in nutrient broth amended with different concentrations of APO (μg/ml). APO was solubilized in 100% ethanol (etho) and appropriate dilutions made to nutrient broth (NB); etho, ethanol control, representing the highest amount of APO added to nutrient broth during the experiment and shown for clarity as each dilution contained a comparable ethanol control; NB, nutrient broth controls

detoxify it within the 2-wk observation period. Knop et al. (2007) reported that *F. verticillioides* could utilize APO as a C-source, but indicated that its growth was reduced by 14% when cultured with 1 μM of APO. This concentration was considerably less than that used here and elsewhere (Macias et al. 2005; Martyniuk et al. 2006; Krogh et al. 2006), which are concentrations reported to occur naturally (Martyniuk et al. 2006). Perhaps the growth reduction observed in *F. verticillioides* by Knop et al. (2007) is reflective of the small amount of APO used. However, statistical significance was not assigned to the 14% reduction observed.

We established earlier that *F. verticillioides* was capable of cleaving the oxazolone ring of BOA, producing AP that is oxidized to HPMA or HPAA, and the latter caused primarily by the inactivity of the pathway control by FDB2 (Glenn et al. 2003). It was not established that the two genetic loci have direct relationships to the accumulation pattern observed by strains of *F. verticillioides*. The two strains of *F. verticillioides* genotypes used here represented two genotypes of 24 random ascospore progeny, and each differ in the number of functional alleles at both loci, designated Fdb1 and Fdb2. Tolerant strains have both alleles at both loci, and sensitive strains are defective either at one or both loci, i.e., FDB1/*fdb2* or *fdb1*/FDB2. Significant amounts of HPAA are produced only by strains of the genotype Fdb1/fdb2, thus, AP is not transformed to HPAA by all strains. Further, neither the natural occurrence nor the toxicity of HPAA to other organisms has been examined. In general, however, wild-type strains of *F. verticillioides* and several *Fusarium* species all show tolerance to most metabolites of BOA (Yue et al. 1998; Glenn 2001; Glenn et al. 2001) with detoxification proceeding from BOA or MBOA resulting in the accumulation of HPMA. The primary focus of concern, therefore, is the transformation of AP to either HPMA, a substance not toxic to most naturally occurring strains of *F. verticillioides*, or to APO.

The transformation of AP to the more biologically active APO has relevance, especially at the ecological level. The ability of *F. verticillioides* to detoxify AP into several nontoxic products led to the tentative conclusion by Glenn et al. (2002) that the benzoxazolinones allelochemicals are probably not effective resistance mechanisms against this fungus. However, interactive microbial activity was not considered relevant. As established here and by others (Gagliardo and Chilton 1992; Fomsgaard et al. 2004; Fritz and Braun 2006), however, APO is an important microbial transformation product of AP, although the interactions are perhaps exceeding complexity as observed here and by others (Macias et al. 2005; Knop et al. 2007).

Our interpretation of the results obtained in this study is that the inhibition of growth of *F. verticillioides* by *B. mojavensis* also prevents the efficient transformation of AP by *F. verticillioides* to the nontoxic HPMA, which is summarized in Fig. 5. The fungus in the presence of the bacterium seems capable of hydrolyzing BOA to AP, but then apparently is unable or at least has a reduced capacity for modifying AP to HPMA, resulting in an accumulation of AP that is either converted by the bacterium or spontaneously oxidized to APO. APO is more toxic to a broad range of microbes, including *F. verticillioides*. *B. mojavensis* is able to tolerate APO, thus an environment is created that favors the bacterium. As the effect is observed to occur some distance on culture plates from the bacterium, there is a suggestion for the production of an inhibitory diffusible substance. We speculate that if indeed an inhibitory substance is produced, it would interfere with the enzymatic function of the second genetic locus, FDB2, resulting in the accumulation of APO (Fig. 5). We do not know if this substance is the same as the fungal growth inhibitor produced in media by the bacterium that inhibits the *F. verticillioides* (Fig. 2a).

This is the first report to show that in the presence of a bacterium on BOA-amended medium there is an increased expression of *in vitro* toxicity to a fungus, which usually has the capability to detoxify BOA into decomposition products. Recently, Knop et al. (2007) demonstrated that detoxification of BOA by *F. verticillioides* was greatly reduced in the presence of the herbicide metolachlor. This information along with our data suggests that biotic and abiotic stress can influence the overall allelopathic interaction with the benzoxazolinones. As APO is the major transformation product of AP by most soil microorganisms, the role of APO and its accumulation by a fungal antagonist is highlighted here with several ecotoxicological possibilities. As *B. mojavensis* is an endophyte, the possible endophytic transformation of BOA into APO offers additional benefits. Further, the long-term stability of APO in soil types (Macias et al. 2005) and the suggestion that it might be the final transformation products by organisms in most soil types (Fritz and Braun 2006) are other features that should enhance the use of benzoxalinones combined with tolerant organisms such as *B. mojavensis* as effective biocontrol agents.

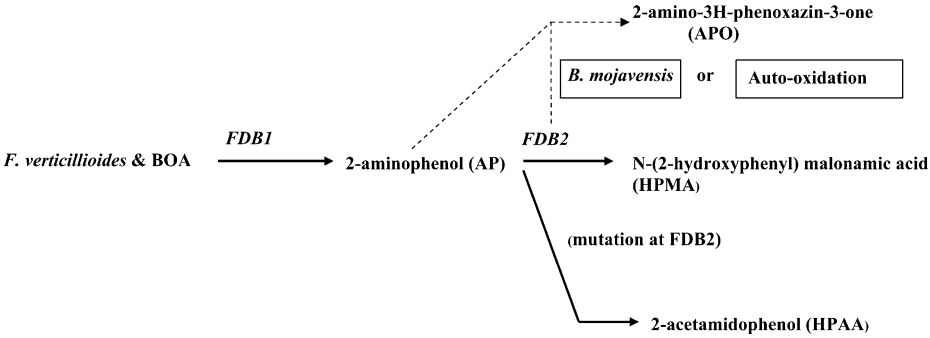


Fig. 5 Theoretical detoxification of BOA by co-cultures of *Bacillus mojavensis* and *Fusarium verticillioides* (modified from Glenn et al. 2003). *F. verticillioides* RRC826 cultured on BOA amended PDA transforms BOA, which involves two functional genetic loci, *FDB1* and *FDB2*, leading to the production of a relatively nontoxic *Fusarium* transformation product, HPMA. However, a mutation at *FDB2* causes the accumulation of a novel product as a result of the acetylation of AP to form HPAA. We theorize that in the presence of the wild-type loci of *F. verticillioides*, *B. mojavensis* interferes with the locus *FDB2*, resulting in an accumulation of APO, which results either from a physical spontaneous oxidation of AP to form APO, or direct transformation by *B. mojavensis* to APO. Under either pathway some HPAA should also accumulate

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Keywords *Phragmites australis* · *Spartina alterniflora* · Root exudation · Allelopathy · Gallic acid · Reactive oxygen species · Microtubule

Introduction

Phragmites australis (Cav.) Trin. ex Steudel, known as common reed, is a perennial graminaceous plant that propagates mainly through rhizomes and, at low frequency, through seeds (Coops and Velde 1995). *P. australis* occurs in every state of the United States except Arkansas (probably present but undocumented) and is predominantly seen along the borders of lakes, ponds, and rivers (Harrington 1964). According to archaeological records, it has been present in the United States for at least 40,000 yr and it is considered a native species (Hansen 1978). However, genetic analysis of *P. australis* has shown that the more aggressive exotic European genotype has been introduced and is displacing the native genotype by invading new areas (Blossey 2002; Saltonstall 2002). *P. australis*'s distribution and relative abundance has increased dramatically over the last 150 yr (Saltonstall 2002). Presently, it represents one of the most abundant plant species in the US coastal wetlands and is considered an indicator of wetland disturbance (Blossey 2002).

Exotic plant invasions into a new ecological niche are often dangerous and are known to threaten ecosystems across the world (Mack et al. 2000). Such invading plant species not only compete with native species for space and resources but also often replace the entire native biodiversity leading to the formation of monocultures (DiTomaso 2000). Of an estimated 50,000 species introduced as exotic species, around 10% are plant species that are reported to have escaped from their natural ecosystems (Pimentel et al. 2000). Such introduced exotic plants often turn invasive and result in devastating effects on the ecosystem and account for huge economic losses (Zedler and Kercher 2004). The introduction of an invasive species to a new habitat creates competition for space and resources with native species. Traditionally, introduced species are more aggressive because of the nonavailability of predators in the new habitat and their ability to produce potent allelopathic chemicals (Bais et al. 2003a; Inderjit et al. 2006). *P. australis* is now considered as one of the major invasive species causing considerable negative impact on native biodiversity (Marris 2005). Human activities that lead to ecological disturbances, stresses such as pollution, changes in hydrologic regimes, and increased soil salinity, have all been proposed as causes for the rapid expansion of *P. australis* (Minchinton 2002). In addition, a number of edaphic factors have been suggested (Chambers et al. 2002).

Root allelopathy is one of the factors responsible for the invasive success of many plant species (Callaway et al. 1999; Callaway and Aschehoug 2000; Bais et al. 2003a,b, 2006). Root allelopathy specifically refers to the secretion of phytotoxins through root exudates of one plant species that suppress the growth of other species in the vicinity (Bais et al. 2003a, 2006). The Eurasian native spotted knapweed (*Centaurea maculosa*), one of the most economically destructive exotic invaders of North America, uses root-secreted chemical warfare and displaces other weeds and crops (Callaway et al. 1999; Callaway and Aschehoug 2000). Use of novel biochemical weapons that function as unusually powerful allelopathic agents or as mediators of novel plant–plant and plant–soil microbial interactions is known as the 'novel weapons hypothesis' (NWH) (Callaway and Ridenour 2004; Inderjit et al. 2006). (±)-Catechin, a root-derived toxin secreted by spotted knapweed, has been reported to have broad-spectrum allelochemical activity against a number of plant

species (Bais et al. 2002, 2003a). Leafy spurge (*Euphorbia esula* L.), another exotic perennial weed of Eurasian origin is known to rhizo-secrete jatropane diterpenes and ellagic acid derivatives that suppress other plants in its vicinity (Qin et al. 2006).

Apart from edaphic and environmental factors, allelopathic interaction among salt marsh plants has been suggested as an explanation for the zonation in the marsh exhibited by *P. australis* and other species (Drifmeyer and Zieman 1979). However, their experiments with *P. australis* rhizosphere washings did not inhibit germination of the two salt marsh plant species, *Distichlis spicata* or *Scirpus robustus*. Three triterpenoids (β-amanacin, taraxerol, taraxeron) and a flavone (tricin) have been identified from the aerial portions of *P. australis* (Ohmoto 1969; Kaneta and Sugiyama 1972). In addition, various phytochemicals have been extracted from *P. communis* (an earlier name for *P. australis*) and tested for their anti-algal effect on different algal species (Li and Hu 2005). The screen identified a phytotoxic effect of ethyl-2-methylacetoacetate against the algal species *Chlorella pyrenoidosa* and *Microcystis aeruginosa* (Li and Hu 2005). None of these chemicals has been tested for allelochemical activity on other seed plant species. To the best of our knowledge, no previous studies have isolated and characterized chemicals from root exudates of *P. australis* nor tested them for potential allelochemical activity. Considering the complexity of isolating and characterizing allelochemicals secreted from the roots into the soil, we addressed this issue by establishing an *in vitro* system, whereby *P. australis* secreted allelochemicals into a growth medium.

We systematically examined root secretions from two *P. australis* genotypes for their phytotoxicity, by following a bioassay-driven approach that used the model plant *Arabidopsis thaliana*. We identified a phytotoxic compound, gallic acid, as the phytochemical responsible for the root phytotoxicity of *P. australis*. We also established that the identified compound is a broad-spectrum rhizotoxin, which is effective against a diverse range of plants. Finally, we demonstrated that gallic acid generates elevated levels of reactive oxygen species (ROS) in the roots of the treated plants, thus leading to ROS-mediated microtubule disruption and complete collapse of the root architecture.

Materials and Methods

Plant Material and Chemicals

Arabidopsis thaliana wild type cultivar Columbia (Col-0) seeds were procured from Lehle Seeds (Round Rock, TX, USA); certified seeds of tobacco (*Nicotiana tabacum*) and mustard (*Brassica juncea*) were purchased from Ritchters Herb Specialists (Goodwood, Ontario, Canada). The GFP fusion line for tubulin-specific kinase was obtained from J.Y. Lee, Delaware Biotechnology Institute, Delaware, USA. Lettuce (*Lactuca sativa*) seeds were obtained from W. Atlee Burpee (PA, USA). Seeds of three native grass species (*Poa sandbergii*, *Festuca idahoensis*, *Koeleria macrantha*) were obtained from R.M. Callaway, University of Montana, Missoula, MT, USA. *Spartina alterniflora* seeds were obtained from Environmental Concern, P.O. Box P, 201 Boundary Lane, St. Michaels, MD, USA. Aseptic cultures of *P. australis* P38 and BB were established in tissue culture and were obtained from the *P. australis* genotype collection at the Halophyte Biotechnology Center, College of Marine and Earth Studies, University of Delaware. The aseptic cultures were used for the collection of root exudates and all other *in vivo* and *in vitro* studies. Gallic acid was obtained from Sigma-Aldrich, USA.

Culture Conditions

Seeds were washed in double-distilled water three times and surface-sterilized with 50% commercial bleach (sodium hypochlorite) for 3–5 min for *A. thaliana* and 5–10 min for all the other seeds followed by 3–4 washes in sterile distilled water. Seeds were cultured on Murashige and Skoog's (1962) (MS) solid medium with 3% sucrose, and allowed to germinate for 5–10 d until roots and shoots emerged. They were incubated at 25±2°C under a 16:8 h L/D regime. Plates were illuminated with cool fluorescent light with an intensity of 24 μmol m^{-2} s^{-1}.

Preparation of Root Exudates

P. australis genotypes, P38 and BB, were previously determined to be exotic (haplotype M) and native (haplotype F) genotypes, respectively, according to the methods of Saltonstall (2003). About 40 new plantlets of *P. australis* genotypes P38 and BB were inoculated into 400 ml 3% MS liquid medium in 1 l Erlenmeyer flasks, and the cultures were incubated on a rotary shaker set at 90 rpm at 25±2°C. Cultures were provided with a 16:8 h L/D photoperiod. After 25 d of incubation, the spent medium with the secreted root exudates was collected and freeze-dried in a lyophilizer (Labconco). Lyophilized powder was used as the source of root exudate for all phytotoxicity assays and for high-performance liquid chromatography (HPLC) fractionation. Lyophilized (2.5 g) powder was dissolved in 10 ml of distilled water and filter-sterilized by passing through a 0.45 μm filter (Millipore). The filtered liquid was used as sterile root exudate stock for all *in vitro* phytotoxicity assays.

In Vitro Phytotoxicity Assays

Three- to 5-d-old *A. thaliana* Col-0 and 5- to 6-d-old tobacco (*Nicotiana tabacum*), lettuce (*Lactuca sativa*), mustard (*Brassica juncea*), and smooth cordgrass (*Spartina alterniflora*) seedlings that had been germinated on MS solid medium with 3% sucrose were transferred to 24-well plates containing 1% MS liquid medium with respective treatments of root exudates. The treatments included 10%, 20%, and 30% (*v*/*v*) root exudates of *P. australis* and 50, 100, and 150 μM gallic acid separately. Control cultures did not receive any root exudates. Another control was set up with 30% sterile water to account for the dilution effect, if any. Similarly, the effect of gallic acid (100 μM) was also studied on three grass species (*P. sandbergii*, *F. idahoensis*, *K. macrantha*). A positive control with 100 μg ml^{-1} (±)-catechin, a known rhizotoxin (Bais et al. 2002), was also included for comparison. Cultures were incubated on a rotary shaker set at 90 rpm maintained at 25±2°C. Plants were illuminated with cool white fluorescent light for a 16:8 h L/D photoperiod. All treatments had six replicates, and the experiment was repeated at two independent times. The experiment was terminated at the end of the 10th d, and the observations were recorded as fresh weight in grams.

Identification of Compounds in Root Exudates

For HPLC analysis, 100 mg of the lyophilized powder of the root exudates were dissolved in 500 μl of 2.5 mM potassium phosphate buffer (pH 2.5) and filtered; 30 μl of the filtrate were injected. The major peaks separated on an Altech organic acid column (Prevail, Organic acid, 5 μm; 150×4.6 mm) were collected and assayed for phytotoxicity *in vitro* as

described earlier. Peaks that showed phytotoxicity were collected and resubjected to HPLC analysis and compared with the standard gallic acid peak to match the retention time. 1 μg μl^{-1} stock solution of standard gallic acid was prepared in 2.5 mM potassium phosphate buffer (pH 2.5) and was used for the comparison and estimation of gallic acid in the root exudates by using the peak areas. HPLC was performed in a Dionex HPLC system, equipped with a photodiode array detector. The separation was performed with an isocratic mobile phase of 2.5 mM potassium phosphate buffer (pH 2.5). Similarly, one additional set of separations was done by using a step-wise gradient of methanol and water as the mobile phase on a C_{18} column (Dionex Acclaim®, Polar Advantage II, 5 μm, 120 Å, 4.6× 150 mm). The methanolic fraction was subjected to electrospray ionization–mass spectrometry (ESI–MS) analysis to trace the molecular ion mass peak in the root exudates for confirmation.

Effect of *P. australis* and Activated Charcoal on Growth of *A. thaliana*

Pots of 40-d-old P38 and BB *P. australis* were seeded with about 50 seeds of *A. thaliana*. The germination and establishment of *A. thaliana* plants were monitored. After 6 wk, observations were recorded in terms of the number of *A. thaliana* plants that survived and established in P38 and BB pots. A set of control pots with only *A. thaliana* plants was also set up, and the number of plants surviving after 4 wk was counted, and the data were expressed as percent survival. A similar experiment was conducted with additional pots with 20% (*w*/*w*) activated charcoal (4–8 mesh; from Sigma) in which P38 and BB plants were grown for 40 d and seeded with 50 *A. thaliana* seeds. Germination and establishment of the *A. thaliana* plants were monitored. At 4 wk, pot germination data were collected and presented as percent survival.

Collection of Soil Cores and Extraction of Gallic Acid from *P. australis* Pots and Natural Stands

About 20 plants of each *P. australis* lines P38 and BB were grown in pots containing commercial plant growth soil mix (procured from Miracle-Gro Lawn Products, Marysville, OH, USA) for about 40 d. Then, soil cores (at least six) were drawn from different random spots in the pot by using a cork borer as a sampling device, and they were sectioned into the top 5 cm, middle 5 cm, and bottom 5 cm depths. For each respective depth, 0.5 g samples were taken from the respective sections and mixed to form a representative composite sample. This was done for each pot. One-half gram of each composite sample was extracted separately with 8 ml of 2.5 mM potassium phosphate buffer (pH 2.5). The extract was centrifuged, the supernatant was filtered, lyophilized to dryness, and redissolved in 0.5 ml of 2.5 mM potassium phosphate buffer (pH 2.5) and used for HPLC analysis. Similarly, representative soil samples were collected from the sites of natural *P. australis* stands from four different locations (three from near Lewes, DE and one from near Darien, GA; respective GPS locations of these sites are listed in the legend to figures). The roots and root bits or damaged roots if any in the soil samples were hand-removed to obtain a clean soil sample without any roots for field samples. Damage to roots in pot sampling was avoided by sampling away from the root core zone. Composite samples were prepared by mixing thoroughly the 10 samples collected from the same location, and about 10 replicates of such samples were obtained for analysis. One gram each of the soil sample was extracted in 10 ml of 2.5 mM potassium phosphate buffer (pH 2.5), the extract was centrifuged, and the supernatant was collected, filtered by passing through 0.45 μM filter, and freeze-dried.

The entire freeze-dried powder was redissolved in 400 μl 2.5 mM potassium phosphate buffer (pH 2.5) and used for HPLC analysis.

In Vitro Assay

An *in vitro* germination assay was conducted to test the effect of root exudate of *P. australis* P38, BB (10%, 20%, and 30% *v*/*v*), and gallic acid (50, 100, and 150 μM). Plates containing the respective treatments were prepared by adding root exudates and gallic acid directly to molten MS solid medium with 3% sucrose. The solidified media in the Petri dishes were seeded with 50 each of surface-sterilized *A. thaliana* seeds. Control plates contained only MS medium without root exudates, and the chemicals were also seeded with 50 surface-sterilized *A. thaliana* seeds. The plates were incubated for 5 d, and the number of seeds germinated was counted, and the data expressed as percent survival. Six replicates per treatment were taken, and the experiment was repeated twice.

Imaging for Reactive Oxygen Species

ROS imaging was done by following a slightly modified protocol of Foreman et al. (2003). Briefly, 4- to 5-d-old *A. thaliana* seedlings germinated on MS solid medium with 3% sucrose were transferred to MS liquid medium with 1% sucrose and pretreated with 20% root exudates of *P. australis* BB, P38 genotypes, and 50 μM gallic acid with or without 500 μM ascorbic acid (AsA) for 30 min; controls did not receive either root exudates or chemicals. After 30 min, 2.5 μM 2′,7′-dichlorodihydrofluorescein diacetate (HB_2-DCFDA) dissolved in DMSO (less than 0.001% final concentration) procured from Invitrogen (USA) was added to the cultures and incubated for 30 min at 4°C. Cultures were washed with 0.1 mM KCl, 0.1 mM $CaCl_2$ (pH 6.0). Washed roots were incubated in MS liquid medium with 1% sucrose for another 30 min at 23±2°C. A time point experiment was also carried out by treating the roots with gallic acid with or without AsA (500 μM) from 0 to 40 min. Roots were imaged by using excitation and emission wavelengths of 488 nm and 522 nm, respectively. The images were of 512×512 pixels with 10 scans of 1 s each in a confocal laser scanning microscope (CLSM) detailed in the following section. Images were captured with either a ×10 or ×40 (with water correction) objective on a Zeiss LSM 510 NLO attached to an Axiovert 200M with an automated stage microscope equipped with Zeiss LSM 510 software.

Effect of *P. australis* Root Exudates and Gallic Acid on Root Cell Microtubules and Root Viability

An experiment similar to that of the *in vitro* phytotoxicity assay was set up by using an *A. thaliana* transgenic line that carried a GFP fusion for a microtubule-specific kinase (CTD-PAPK1-GFP). Plants were incubated for 5 h with root exudates (20% *v*/*v*) and gallic acid (50 μM) separately with different treatments. Treatments included gallic acid, gallic acid plus 500 μM AsA, a control without any chemical, and another control with only AsA. Roots were imaged for microtubule arrangement in control and treated plants by using a confocal scanning laser microscope (CSLM). A time point experiment was also conducted with gallic acid treatment with or without AsA and a positive control with H_2O_2 (10 μM). The treated roots were imaged at different time intervals from 0 to 40 min for microtubule architecture. Images were captured with a ×40 objective (water correction lens) on a Zeiss LSM 510 NLO attached to an Axiovert 200M with an automated stage microscope

equipped with Zeiss LSM 510 software. Similarly, a viability assay was also conducted by using the *A. thaliana* plants treated only with root exudates and gallic acid. Roots were visualized by staining with fluorescein diacetate (FDA) and imaged with CSLM.

Effect of AsA on *in vitro* Survival of *A. thaliana*

About 3- to 5-d-old *A. thaliana* Col-0 seedlings germinated on MS solid medium with 3% sucrose were transferred to 24-well plates that contained 1% MS liquid medium with 50, 100, and 150 μM gallic acid with or without 500 μM AsA. Cultures were incubated on a rotary shaker set at 90 rpm maintained at 25±2°C. Plants were illuminated with cool white fluorescent light for a 16:8 h L/D photoperiod. All treatments had six replicates, and the experiment was repeated on two independent occasions unless otherwise mentioned. The experiment was terminated at the end of the 10th d, and the observations were recorded as fresh weight in grams.

Statistical Analysis

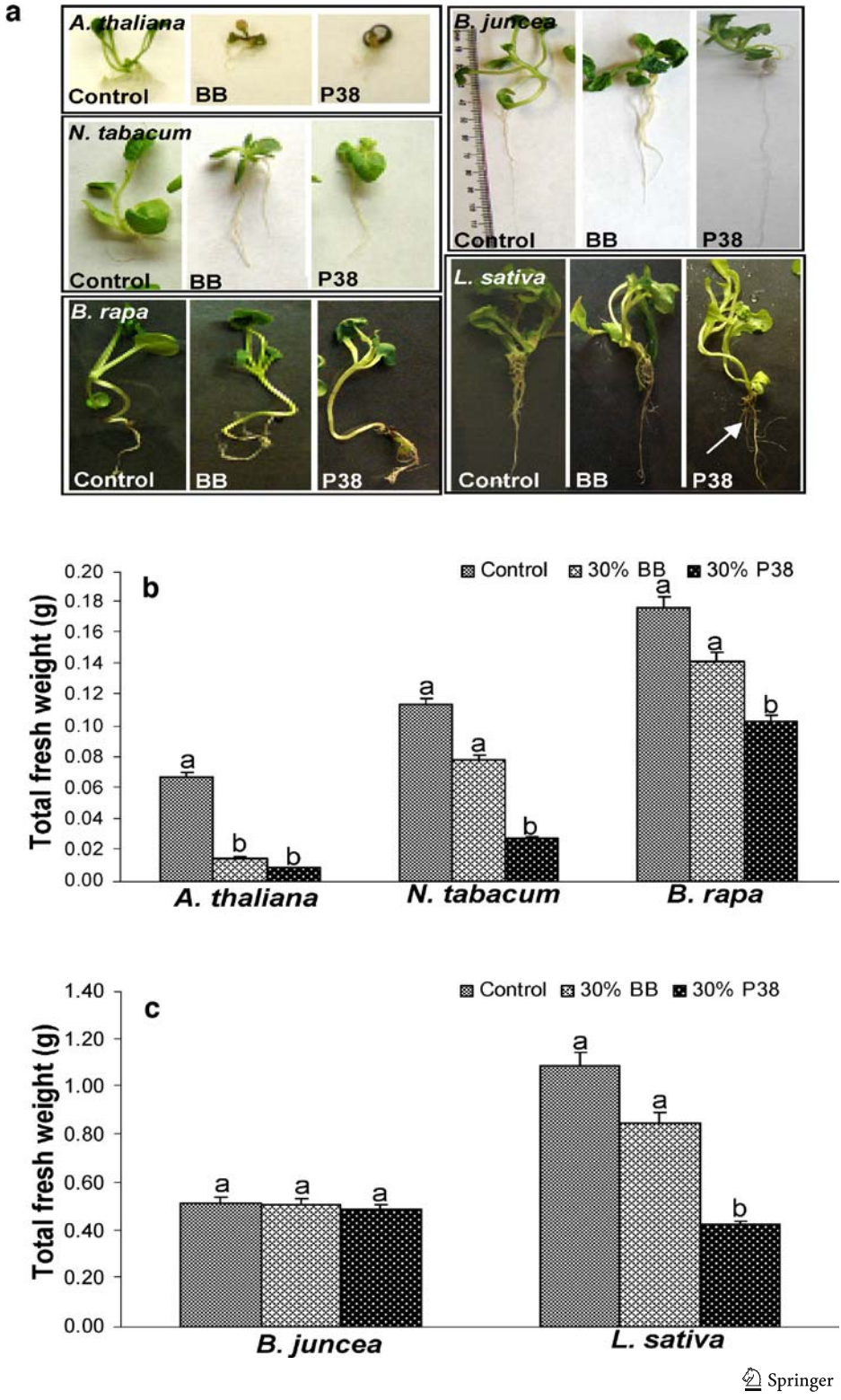
Data on total fresh weight of the plants, the number of plants that survived, and persistence of gallic acid in the soil were averaged from two separate experiments and further analyzed for variance using the Microsoft Excel XP version, followed by a Student's *t* test (Sokal and Rolf 1995). The data means were considered significantly different at the probability of $P<0.05$.

Results

P. australis Root Exudates are Phytotoxic

The phytotoxicity assay results show a severe inhibitory effect of the *P. australis* root secretions on the seedling growth of *A. thaliana* (Fig. 1a). Among the other species tested, the severe inhibitory effect was observed for tobacco (*N. tabacum*), turnip (*B. rapa*), and lettuce (*L. sativa*) (Fig. 1a–c). It is surprising to note that the root exudates did not show any significant growth effect on mustard (*B. juncea*) seedlings. There was a highly significant ($P<0.05$) reduction of biomass production, as measured in terms of total fresh weight (Fig. 1b and c). Root death generally started from the root tip continuing progressively toward the central elongation zone (data not shown). The roots turned completely dark by the end of the 10th d after the crude exudate treatment (Fig. 1a). However, the viability staining of the *A. thaliana* roots with FDA post 24-h treatment with root exudates revealed that the roots lost viability by 24 h after treatment at higher concentrations (30%) of P38 root exudates (Supplementary Fig. 1) as indicated by the reduction in the green fluorescence. The effect was more severe in the case of the plants treated with root exudates from exotic line P38 when compared to that of the native BB (Fig. 1; Supplementary Fig. 1).

Fig. 1 Effect of root exudates of *P. australis* native (*BB*) and exotic (*P38*) genotypes on the growth and development of *A. thaliana*, *N. tabacum*, *B. rapa* (**a** and **b**), *B. juncea*, and *L. sativa* (**a** and **c**) on the 10th d after treatment. The data represent the amount of growth inhibition by root exudates in terms of fresh weight accumulation and complete suppression (*arrow*), collapse, and death of the roots (values are presented as the mean±SD, *N=6*). *Different letters on the bars* are used to indicate means that differ significantly ($P<0.05$)


a
A. thaliana
Control
BB
P38
N. tabacum
Control
BB
P38
B. rapa
Control
BB
P38
B. juncea
Control
BB
P38
L. sativa
Control
BB
P38
b
Control
30% BB
30% P38
Total fresh weight (g)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
a
b
b
a
a
b
a
a
b
A. thaliana
N. tabacum
B. rapa
c
Control
30% BB
30% P38
Total fresh weight (g)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
a
a
a
a
a
b
B. juncea
L. sativa

P. australis Inhibits the Growth of *A. thaliana* Seedlings *in vivo*

The root exudates of *P. australis* demonstrated a severe growth inhibitory effect on *A. thaliana* plants grown *in vitro*. Therefore, we speculated that if the root-secreted component is involved in this process, the effect should also be seen *in vivo*. Hence, to mimic conditions that are more realistic, we grew the *P. australis* plants in pots containing plant growth soil mix. After the establishment of the *P. australis* plants for about 40 d, the pots were seeded with a known number of *A. thaliana* seeds (N=50). Germination and establishment of the *A. thaliana* seeds was studied for 6 wk. In line with our *in vitro* results, 6 wk after seeding, complete inhibition of *A. thaliana* seedling growth occurred in the pots with the exotic *P. australis* line P38 (Fig. 2a and b). *A. thaliana* plants grown with P38 showed 97% mortality (Fig. 2a and b) when compared to control pots, which were seeded with *A. thaliana* plants alone. The results also demonstrate greater mortality of the

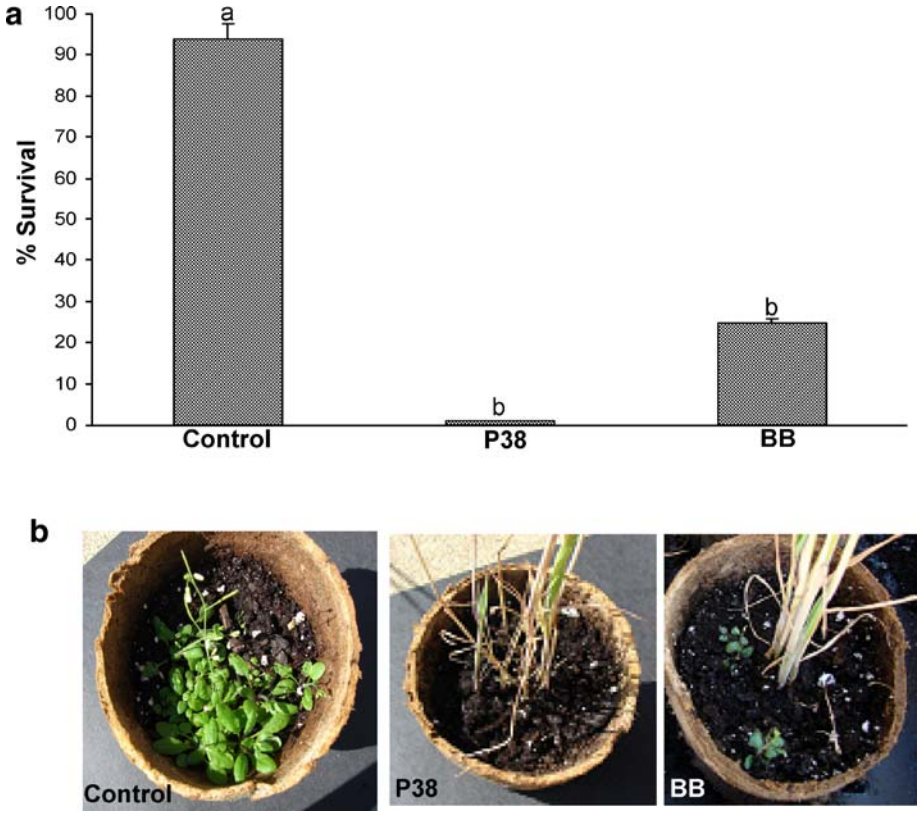


Fig. 2 Effect of *P. australis* (*P38*, exotic; *BB*, native) on the *in vivo* (in pot) germination, establishment, and survival of *A. thaliana* (**a** and **b**). Pots with 40-d-old *P. australis* plants were seeded with a known number of *A. thaliana* seeds. The germination and establishment of the *A. thaliana* seedlings was monitored for 6 wk, and the post 6-wk seeding data are expressed as the % survival (values are presented as the mean±SD, N=4). *Different letters on the bars* are used to indicate means that differ significantly (P<0.05)

A. thaliana plants grown with P38 (exotic) seedlings than those grown with BB (native) seedlings (Fig. 2a and b). These observations together with the *in vitro* results confirm the involvement of a root exudate component in the phytotoxic effect of *P. australis* on susceptible plants.

Application of Activated Charcoal Confirms the Allelopathic Activity

To confirm the involvement of root allelopathy in *P. australis*, we predicted that if we introduced an exogenous component that makes the root-secreted compounds unavailable for plant uptake, it would result in the survival of the susceptible plants. Activated charcoal has been previously reported to act as a sink that adsorbs secreted root exudates, thus making them unavailable for plant uptake (Callaway and Aschehoug 2000; Bais et al. 2003a). Accordingly, we prepared a potting soil mix with 20% activated charcoal, planted *P. australis* plants, and allowed them to grow. After 40 d, the pots with and without activated charcoal were seeded with about 50 seeds of *A. thaliana* each. The germination and establishment of the seedlings was monitored, and the observations were recorded after 4 wk. The post 4-wk results presented in Fig. 3 show a significant recovery of the *A. thaliana* growth in P38 plants with activated charcoal ($P<0.05$) compared to the P38 plants

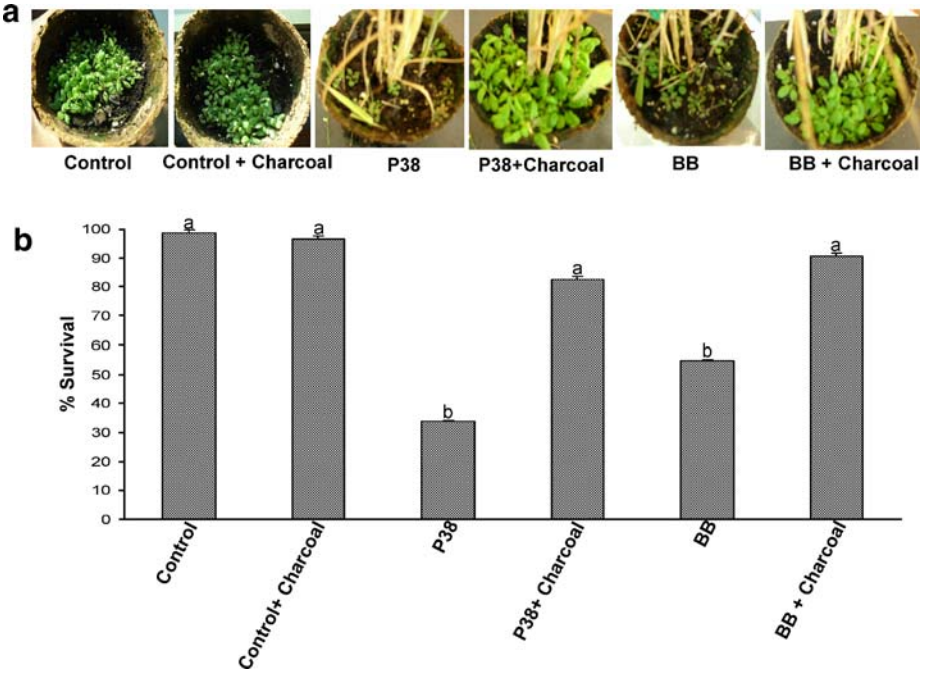


Fig. 3 Effect of activated charcoal (20% *w*/*w*) on the *in vivo* susceptibility of *A. thaliana* to *P. australis*. The pots of 40-d-old *P. australis* grown in plant growth soil mix containing 20% activated charcoal (*w*/*w*) were seeded with 50 *A. thaliana* seeds. The germination and establishment of the seeds was monitored for 4 wk. **a** Shows the more robust and vigorous growth and establishment of *A. thaliana* seedlings in the P38 (exotic) and BB (native) pots with activated charcoal. **b** Shows the plant survival in terms of the number of seeds germinated and established under different treatment conditions (values are presented as the mean±SD, $N=3$). *Different letters on the bars* are used to indicate means that differ significantly ($P<0.05$)

without activated charcoal. It is interesting to note that the *A. thaliana* cocultured with P38 plants with activated charcoal were more robust and fitter than control plants or control plants plus charcoal (Fig. 3). A similar response was also observed with the BB plants. Activated charcoal alone did not have any inhibitory effect on the germination and establishment of *A. thaliana* (Fig. 3). These data confirm that root exudates, in particular, have a role in the allelopathy of *P. australis*.

2,3,5-Dihydroxy Benzoic Acid (Gallic Acid) is the Phytotoxic Compound in the Root Exudates

A previous report suggested the involvement of an anti-algal effect by *P. communis* whole plant extracts (Li and Hu 2005). The authors demonstrated a phytotoxic effect of ethyl-2-methylacetoacetate against the algal species *Chlorella pyrenoidosa* and *Microcystis aeruginosa* (Li and Hu 2005). As our previous results showed the presence of a phytotoxic principle in crude *P. australis* root exudates, we adapted HPLC separations to purify the active compound. Purification and characterization of the active phytotoxic compounds were performed as per the published protocol (Bais et al. 2002, 2003a,b; Stermitz et al. 2003; Qin et al. 2006). We found that the phytotoxic activity was limited to one isolated peak, which was identified as gallic acid by HPLC–MS scans (Supplementary Fig. 2). We also analyzed root secretions by HPLC analysis for the presence of ellagic acid (EA), and ethyl-2-methylacetoacetate (EMA). It is interesting to note that we were able to detect only gallic acid in the root exudates. The later two chemicals were either absent or not present in concentrations detectable by HPLC. The presence of the gallic acid peak was confirmed by comparing the representative retention time with that of the standard gallic acid (Supplementary Fig. 2a and b). The presence of gallic acid was also confirmed by doing an ESI–MS analysis for gallic acid in the root exudates. The ESI–MS molecular ion mass peak for standard gallic acid (Supplementary Fig. 2c) and P38 (Supplementary Fig. 2d) exudates are shown. However, root exudates did not show the presence of a molecular ion mass of either ellagic acid or ethyl-2-methylacetoacetate, as reported earlier for the whole plant extracts (data not shown).

Gallic Acid Inhibits the Growth of *A. thaliana* and Other Species

After the identification of gallic acid in the root exudates, we determined that it is phytotoxic by testing standard gallic acid. The results (Fig. 4a–d; Supplementary Fig. 3) showed a severe phytotoxic effect on *A. thaliana* and other plant species, including the closely associated native species *S. alterniflora* and some of the native upland grass species studied with 100 μM gallic acid as LC_{50} ($P<0.05$). It is interesting to note that gallic acid did not show any phytotoxic effect on the seedlings of rice (*Oryza sativa*) or wheat (*Triticum aestivum*) (data not shown). Mortality of the plant was due mostly to the complete suppression of plant growth as a result of the death of the primary root similar to the effect shown by the *P. australis* P38 root exudates (Fig. 1a). Concomitantly, neither ellagic acid nor ethyl-2-methylacetoacetate showed any growth inhibitory activity or rhizotoxicity on *A. thaliana* plants (data not shown). The toxic effect of gallic acid on *A. thaliana* roots was further confirmed by the complete loss of viability of the treated roots as shown by the FDA staining (Supplementary Fig. 1). The treatment with the 100-μM gallic acid resulted in the complete loss of fluorescence when compared to the untreated control; these data correlated well with the observation of the 100-μM gallic acid as LC_{50} for *A. thaliana*.

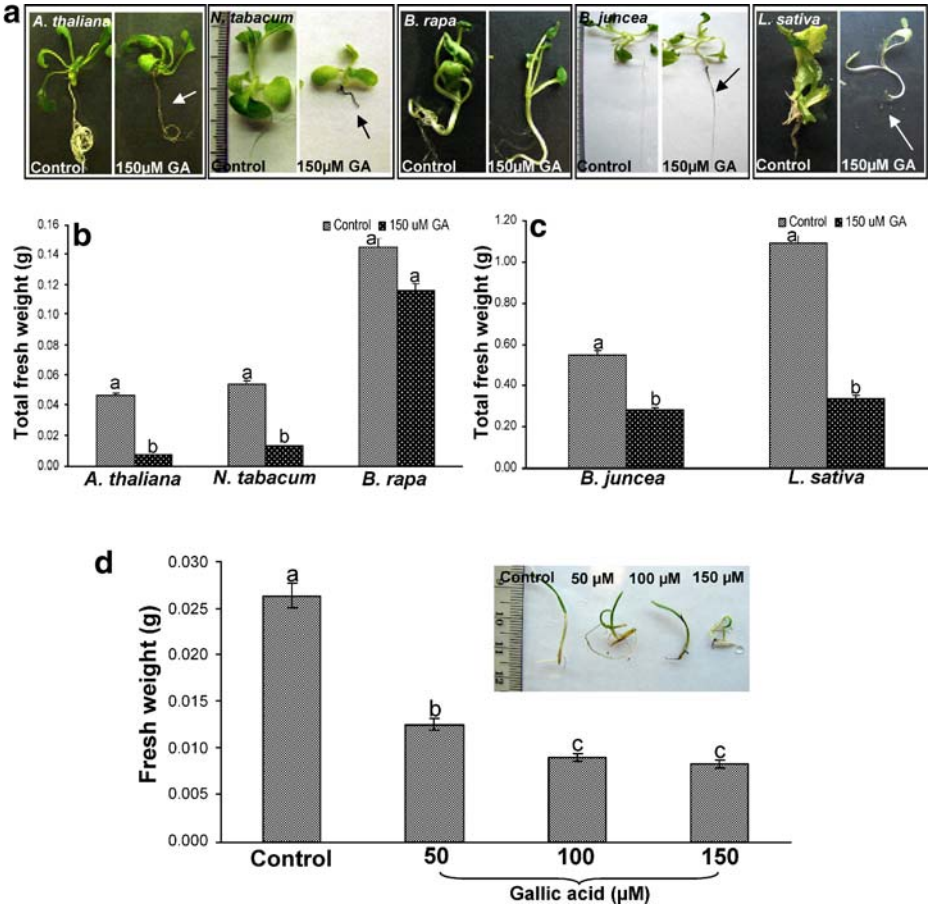


Fig. 4 Effect of gallic acid (*GA*) on the growth and development of *A. thaliana*, *N. tabacum*, *B. rapa* (**a** and **b**), *B. juncea*, *L. sativa* (**a** and **c**), and *Spartina alterniflora* (**d**) on the 10th d after treatment. Gallic acid was dissolved in DMSO and diluted with water to achieve the final concentration of DMSO (less than 0.0001%) and administered (150 μM) to the media containing 3–5-d-old seedlings (see the "Materials and Methods" section). The 24-well plates were subsequently incubated under a 16-h light and 8-h dark photoperiod on an orbital shaker set at 90 rpm at 25±2°C. The data represent the amount of growth inhibition by root gallic acid in terms of severity of killing the roots (**a**; *arrows*) and fresh weight accumulation (values are presented as the mean±SD, $n=6$). *Different letters on the bars* are used to indicate means that differ significantly ($P<0.05$)

Gallic Acid persists in *P. australis* Rhizospheric Soil

As gallic acid is exuded under controlled laboratory conditions, we tested for its presence under realistic rhizospheric conditions. The results (Fig. 5) show the persistence of significantly higher gallic acid ($P<0.05$) in the middle zone (5–10 cm from the top soil) of the pot followed by the top (5 cm) and bottom (10–15 cm from the top soil) zones. In addition, persistence of a significant concentration of gallic acid was also observed in the rhizospheric soils of *P. australis* natural stands from three different locations fringing Delaware Bay near Lewes, DE and one from near Darien, Georgia (Fig 5b, see legend for GPS coordinates). These data further confirm gallic acid as the probable phytotoxic

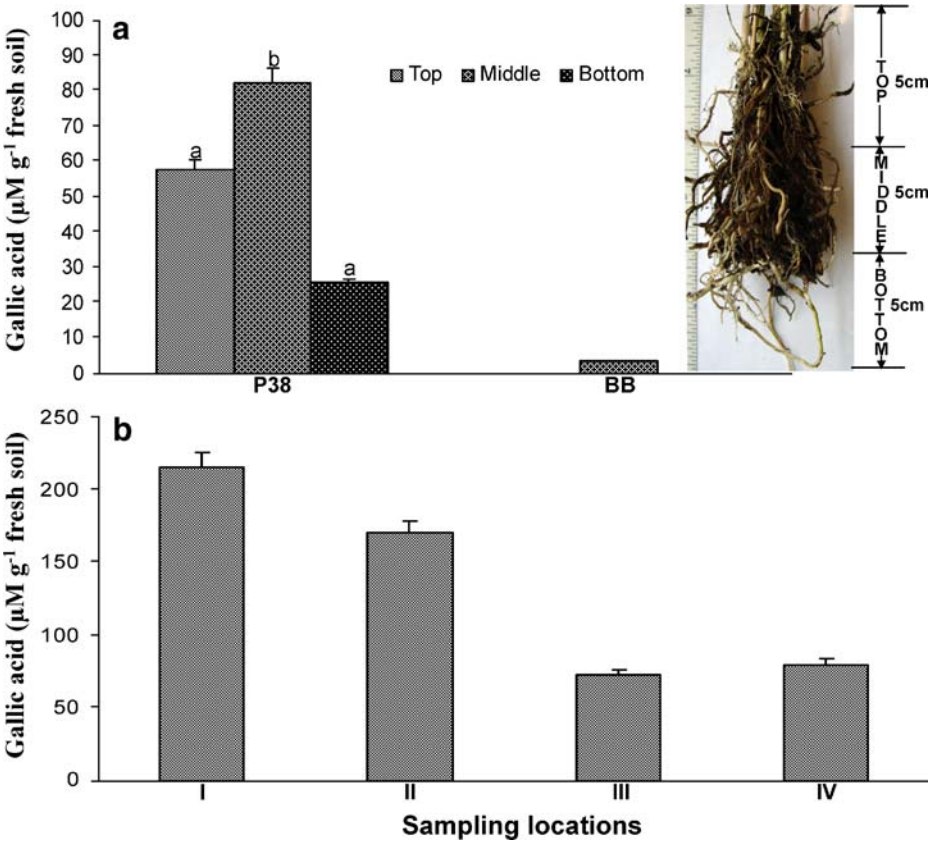


Fig. 5 **a** Secretion and persistence of gallic acid in the rhizospheric soil of *P. australis* (P38=exotic; BB=native) sampled at different depths and estimated by HPLC analysis (see the "Materials and Methods" section). *Inset*: Pictorial depiction of three different depths at which soil cores were collected. *Different letters on the bars* are used to indicate means that differ significantly ($P<0.05$). **b** Gallic acid levels in the soils sampled from natural stands of *P. australis* (all exotic-P38 genotype) from four different locations, three from near Lewes, Delaware [*X*-axis; *I* location I (latitude=38.76604; longitude=75.16674; N38°, 45.9624; W075° 10.0044), *II* location II (latitude=36.78106; longitude=75.15268; N38°, 46.8636; W075° 9.1608), *III* location III (latitude=33.66604; longitude=75.13434; N38°, 47.9624; W075° 11.0044)] and one from near Darien, Georgia [*X*-axis; *IV* location IV ((31° 18′ 43.80″ N, 81° 27′ 26.21″ W)] (values are presented as the mean±SD, $N=10$)

principle secreted through root exudates. It is interesting to note that the data show that *P. australis* plants grown in pots with artificial soil secretes approximately twofold less gallic acid compared to the *P. australis* plants grown in the natural environment. This difference in secretion potential of gallic acid may be because of differences between the edaphic factors in the marsh and the microcosms.

Effect of *P. australis* Root Exudates and Gallic Acid on Germination of *A. thaliana* Col-0 seeds

As most of our phytotoxicity assays involved the use of 5- to 10-d-old seedlings, we questioned whether root exudates and gallic acid have any effect on seed germination. We

studied the germination of *A. thaliana* seeds on root exudates and gallic acid plates. The result (Supplementary Fig. 4) indicates that neither root exudates nor gallic acid appeared to inhibit seed germination. However, root exudates and supplemented gallic acid killed the just emerged *A. thaliana* seedlings (Supplementary Fig. 4a–c). The deleterious effect of root exudates and gallic acid on *A. thaliana* seedlings was concentration-dependent (Supplementary Fig. 4a–c). Furthermore, the seedlings on gallic acid plates initially appeared more robust than controls; this might be an immediate resistance response of the plant to the toxin. However, the seedlings on the gallic acid medium showed severe reduction in root length when compared to control plants, which eventually lead to the death of the plants (Supplementary Fig. 4a and c). These results together suggest that gallic acid functions as a post emergent rhizotoxin. However, there is, of course, also a possibility that root exudates contain additional toxins that kill plants immediately after germination. This needs to be investigated.

Gallic Acid and *P. australis* Root Exudates Generate Elevated Levels of ROS and Disrupt the Microtubule Networks

As gallic acid is a phenolic compound, and such compounds are known to generate ROS (Muzandu et al. 2005), we hypothesized that it might trigger ROS generation in the exudates- and gallic acid-treated roots. To test this hypothesis, we imaged the roots of the wild type *A. thaliana* (Col-0) plants by a fluorescent H_2DCFDA (Foreman et al. 2003) staining method under three different treatments: a control, plus gallic acid, plus both gallic acid and AsA, a strong ROS quencher (Chen and Gallie 2005). Similarly, imaging was also done with the root exudate-treated *A. thaliana* roots. H_2DCFDA is a cell-permeant dye that is activated specifically in response to ROS, and the intensity of fluorescence is proportional to the amount of ROS generated (Foreman et al. 2003). The post 5 h imaging data showed the production of elevated levels of ROS on the roots treated with 50 μM gallic acid when compared to the untreated control *A. thaliana* roots (increased fluorescence in the panel) (Supplementary Fig. 5). The generated ROS was completely quenched when the roots were treated with 500 μM AsA. A similar response was also recorded with *P. australis* root exudates (data not shown). Our goal was to gain insight into the mechanism involved in the phytotoxic role of gallic acid at the cellular level. We suspected that gallic acid-generated ROS and the associated biochemical change on the root surface might result in other downstream processes that lead to the complete collapse of the roots. Considering these aspects, we hypothesized that gallic acid might disrupt the microtubule network of the cell through a ROS-mediated process. To test this hypothesis, we used an *A. thaliana* transgenic line with Col-0 background possessing a GFP-tagged microtubule specific kinase, which specifically labels microtubules. Five-d-old *A. thaliana*-35S: GFP-PAPK1 seedlings germinated on MS solid medium with 3% sucrose were treated with 50 μM gallic acid (the lowest concentration of gallic acid was selected to have a sublethal effect). Post 5 h treated roots were imaged with a confocal microscope. The result (Supplementary Fig. 5) shows that the lowest concentration of the gallic acid used resulted in the complete disruption of the microtubule network. A similar effect was also observed with root exudates (data not shown). With both root exudates and gallic acid treatment, the microtubule assembly appeared to be dismantled and formed localized aggregates of microtubule fibers. However, the effect was reversed when the roots were treated with 500 μM AsA along with 50 μM gallic acid. These data together with the other results suggest that gallic acid acts on the *A. thaliana* roots through ROS-mediated disruption of the microtubule network.

To track the pace of the effect of gallic acid on *A. thaliana* roots, we conducted a time point experiment and imaged the roots for both the generation of ROS and corresponding microtubule disruption. The results showed that by 10 min after treatment, ROS was generated on the root surface and increased further over the time period (Fig. 6a). Correspondingly, the disruption of the microtubule assembly also appeared to be initiated by 10 min, and it reached the maximum level by the 40th min (Fig. 6b). The destruction of microtubules was severe by 40 min, which resulted in the appearance of disassembled microtubules as small florescent specs on the roots (Fig. 6b). To check the involvement of ROS, we used 10 μM H_2O_2 as a positive control and imaged the microtubule network. H_2O_2 is an important ROS and is known also to induce other ROS species (Bolwell et al. 1995). H_2O_2-induced ROS showed a similar and more severe effect than gallic acid and resulted in the complete destruction of the microtubule assembly within 10–20 min of treatment (Fig. 6c). The H_2O_2 at 10 μM showed no inhibitory effect on the roots of *A. thaliana* (data not shown). Treatment of roots with 500 μM AsA along with gallic acid resulted in complete quenching of the gallic acid-generated ROS on the root surface (Fig. 6d). The treatment of roots with AsA along with gallic acid suppressed the negative effect of gallic acid on microtubules and restored the control phenotype of the microtubule network (Fig. 6e). These results established conclusively that gallic acid acts on *A. thaliana* roots through the generation of elevated levels of ROS that lead to destruction of the microtubule assembly and, finally, to the collapse of the root architecture.

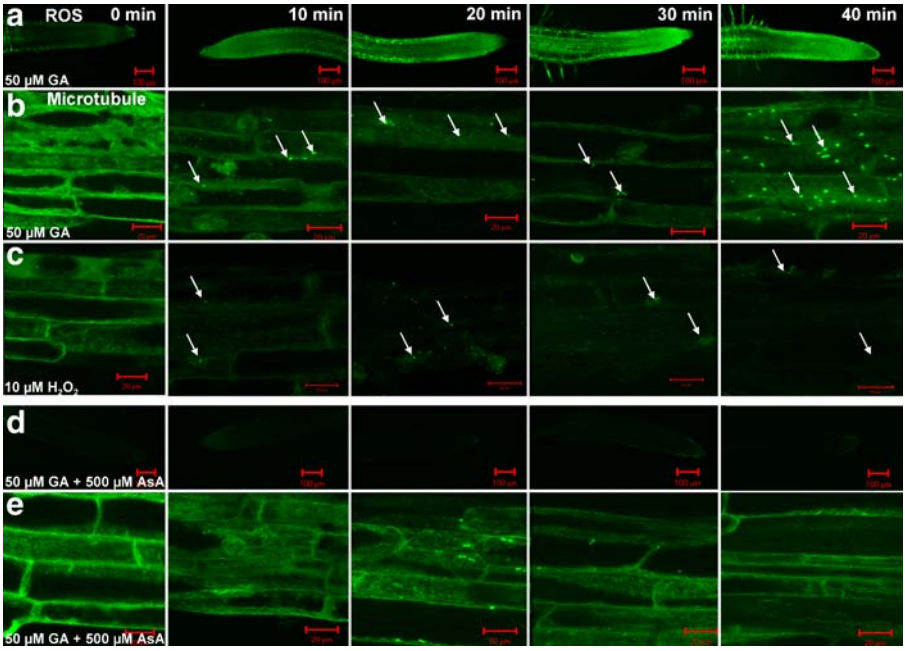


Fig. 6 Induction of root surface reactive oxygen species (*ROS*) by gallic acid (*GA*) (50 μM) (**a**), effect of 50 μM gallic acid (**b**), and 10 μM hydrogen peroxide (**c**) on root cell microtubule architecture over a 40-min time period. Effect of combined treatment of 50 μM gallic acid with 500 μM AsA on root surface ROS generation (**d**) and root cell microtubule architecture (**e**) over a 40-min time period. The images are confocal scanning laser micrographs and each image is representative of the roots of at least six independent plants analyzed and imaged. (Scale bars=100 μM for ROS images and 20 μM for microtubule images). See the "Materials and Methods" section for the details on microscopy and staining methodology

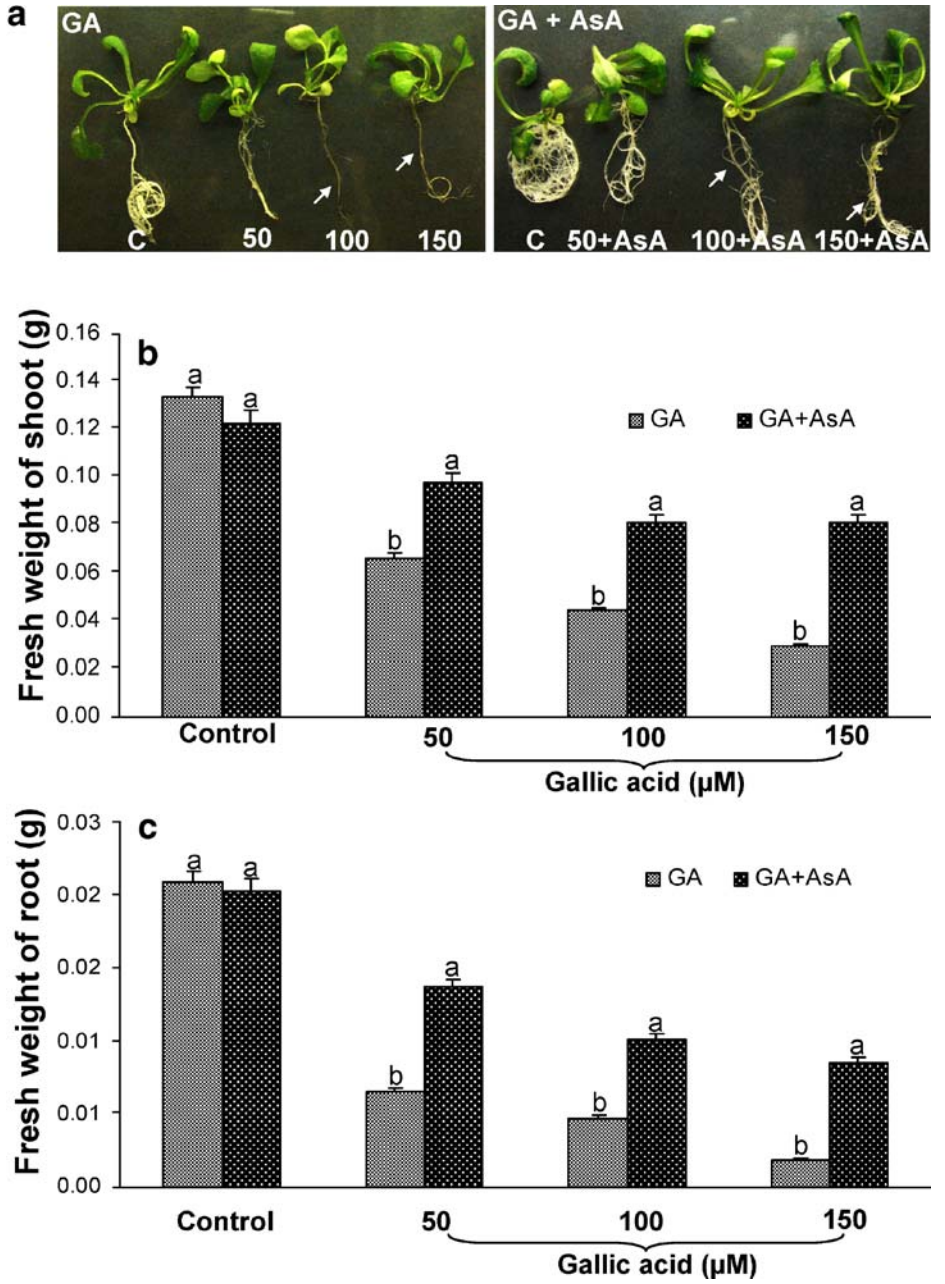


Fig. 7 Role of AsA (500 μM) in the recovery of *A. thaliana* seedlings from gallic acid (GA) (50–150 μM) toxicity *in vitro*. 50–150 μM of gallic acid with or without 500 μM AsA (dissolved in water and filter-sterilized using 0.45 μm filter) were administered to the culture medium holding 5-d-old *A. thaliana* seedlings. The cultures were then incubated under a 16:8 h L/D photoperiod on an orbital shaker set at 90 rpm at 25±2°C. **a** Shows the completely collapsed, killed, and darkened roots of gallic acid-treated *A. thaliana* and the mostly recovered roots when treated with AsA (indicated by *arrows*). **b** and **c** Show the growth and recovery of the *A. thaliana* plants subjected to combined treatment of gallic acid and AsA post 10 d in terms of the production of fresh weight of shoot (**b**) and root (**c**) (values are presented as the mean± SD, N=6). *Different letters on the bars* are used to indicate means that differ significantly (P<0.05)

AsA, a ROS Quencher, Reverses the Effect of Gallic Acid

Our observations presented in the earlier sections established that the phytotoxicity of gallic acid on *A. thaliana* and other species is because of ROS-mediated destruction of the microtubule network of root cells. We verified this by the inclusion of AsA in gallic acid treatment. We set up an experiment that used 5-d-old *A. thaliana* seedlings treated with different concentrations of gallic acid (50–150 μM) with or without AsA (500 μM). The results show that the treatment with AsA resulted in more than 80% recovery from the effect of gallic acid (Fig. 7). The AsA-treated roots appeared normal and similar to the controls when compared to the darkened and dead roots of gallic acid-treated *A. thaliana* (Fig. 7a). Furthermore, greater than 80% recovery in growth in terms of fresh weight of the roots and shoots was observed with the plants treated with AsA along with gallic acid when compared to plants treated with gallic acid alone (Fig. 7b and c).

Discussion

Plant–plant chemical communication and perception strategies are sophisticated but only partly understood. Among the different interactions, the suppressive interaction of a class of chemicals secreted by one plant against neighboring plants (allelopathy) have been implicated in the invasiveness of many exotic weedy species (Callaway and Aschehoug 2000; Bais et al. 2006; Inderjit et al. 2006). *P. australis* is one of the dominant colonizers of U.S. wetland marshes and exhibits invasive behavior by virtually replacing the entire native vegetation in its niche (Lynch and Saltonstall 2002). Genetic analysis of *P. australis* has revealed the introduction of more aggressive genotypes from Europe (Saltonstall 2002). However, in some locations, such as the Lake Superior wetlands, it is the native genotype that is exhibiting invasive behavior, thus complicating the debate over *Phragmites* control if the invader is a native (Wills and Birks 2006). In this communication, through a series of biochemical and cell signalling studies, we report for the first time a root-secreted rhizotoxic compound that is implicated as one of the factors for the invasiveness of *P. australis*. Root exudates of *P. australis* P38 showed strong rhizotoxicity on different tested plants including the model plant *A. thaliana*. The rhizotoxicity observed by both *in vitro* and in pot experiments under greenhouse conditions, supported by increased survival of *A. thaliana* plants in the presence of activated charcoal, indicate the involvement of root exudate-derived allelopathy. Root exudate-mediated allelopathy has been reported in a number of species (Callaway et al. 1999; Callaway and Aschehoug 2000; Bais et al. 2006; Inderjit et al. 2006). Root exudates from *in vitro* grown spotted knapweed (*C. maculosa*) have exhibited phytotoxicity on a number of weedy species including the model plant *A. thaliana* and crops such as wheat (*Triticum aestivum*) and tomato (*Lycopersicon esculentum*) (Bais et al. 2002). In these cases, plants showed wilting symptoms before senescence with reduced shoot and root differentiation (Bais et al. 2002). Similarly, *P. australis* root exudates induced severe rhizotoxicity, leading to the inhibition of root growth with the whole root eventually turning dark and collapsing. Often, such root exudate-mediated toxicity has been attributed to one or a few potential phytotoxins (Bais et al. 2002, 2003b; Stermitz et al. 2003; Qin et al. 2006).

Gallic acid was highly phytotoxic to *A. thaliana* and other plant species tested. It particularly induced rhizotoxicity similar to crude root exudates leading to severe inhibition of root growth and disrupting the total root architecture. Gallic acid, a phenolic compound found in a number of dicot species (e.g., oak, birch, tea, grape, and sumac) (Barman and

Rai 2000; Kraus et al. 2003), is a main constituent of gallotannins. Although, the gallic acid biosynthetic pathway is still debated, it has been shown that gallic acid is synthesized directly from its precursor 5-dehydroshikimate in the leaves of *Rhus typhina* (Werner et al. 2004). Although, the presence of gallic acid has been shown in monocots (Iqbal et al. 2003; Li and Hu 2005), its biosynthetic pathway has not been deduced in these species. It may be because of an altogether different but specific biosynthetic pathway in monocots. The presence of other phenolic acids such as ellagic acid in *P. communis* (former name for *P. australis*) supports this view (Li and Hu 2005). Gallic acid present in the whole plant ethanolic extracts of *P. communis* was negated for any allelopathic effect on two algal species studied (Li and Hu 2005). Ethyl acetate fractions of buckwheat (*Fagopyrum tataricum*) showed the presence of gallic acid and a strong phytotoxic effect on selected plant species (Iqbal et al. 2003). The allelopathic effect of gallic acid has been reported in a number of other species (Weidenhamer and Romeo 2004). In our study, gallic acid showed a severe rhizotoxic effect on the monocot, *S. alterniflora*, a native salt marsh grass often displaced by *Phragmites*, and on the native upland monocot grasses *P. sandbergii*, *F. idahoensis*, and *K. macrantha*, but it is interesting to note that it has no effect on crop species such as rice and wheat (data not shown). The specificity of gallic acid toxicity against various monocot and dicot species should be studied. Its effect on some monocots, including *S. alterniflora*, suggests an allelopathic role in salt marshes. The vast majority of salt marshes overrun by *Phragmites* consist of monocots specifically belonging to the genus *Spartina* (Burke et al. 2002). Our future research will check toxicity on a wide array of both freshwater and saltwater marsh plants.

The phytotoxicity of gallic acid exhibited by the treated roots appears to have been triggered by elevated levels of ROS. This conclusion is supported by the observation that gallic acid treatment resulted in the production of elevated levels of ROS. As the gallic acid treatment caused the collapse of the roots, we suspected that its role was in disrupting the microtubule architecture. Accordingly, we imaged the gallic acid-treated roots of *A. thaliana*-CTD-PAPK1-GFP, a transgenic line carrying a specific GFP tag for visualizing microtubule architecture. This confirmed that increased microtubule disruption was associated with increased ROS levels over a time period. When other plants were incubated with AsA, a ROS quencher and antioxidant (Chen and Gallie 2005), they showed little or no microtubule disruption. Our gallic acid-treated plants exhibited a considerable amount of recovery when incubated with AsA. It is interesting to note that the phytotoxin, (±)-catechin, secreted through the root exudates of *C. maculosa*, was previously shown to trigger a wave of ROS, which led to the Ca^{2+} signaling cascade triggering genome wide changes in gene expression, ultimately to the death of the root system in *A. thaliana* (Bais et al. 2003a; Weir et al. 2003, 2006; Prithiviraj et al. 2007). The phytotoxic effect of gallic acid involved ROS generation and ultimately led to microtubule disruption in the susceptible plant *A. thaliana*. Our results indicate microtubule disruption as the direct effect of ROS generated by gallic acid. However, further studies are needed to establish that genome wide changes upon gallic acid administration lead to gene expression variations corresponding to marker ROS and microtubule genes.

ROS has been implicated in the signaling and induction of various plant processes from pathogenesis (Torres et al. 2002) to various other stimuli such as gravity response, abscissic acid, auxin, gibberellic acid, UV-B light, and nodulation (nod) factors (Zhang et al. 2001; Mackerness et al. 2001; D'Haeze et al. 2003). An important question is how do ROS modify the downstream targets that lead to phytotoxicity? The response reported in the present study might be occurring through ROS-mediated modification of the downstream signaling proteins, thus leading to altered gene expression and cell death. ROS generated by

phenols have been implicated in the microtubule destruction and associated cell death in human cancer cell lines (Makoto et al. 2000). A polyphenol, phenol [1-(2,4-dihydroxyphenyl)-3-hydroxy-3-(4′-hydroxyphenyl) 1-propanone (beta-hydroxy-DHP)], isolated from licorice root (*Glycyrrhiza glabra*) has been reported to inhibit microtubule assembly in human breast and prostate cancer cell lines (Rafi et al. 2002). However, to the best of our knowledge, there are no reports of a plant rhizo-secreted toxin damaging the microtubule network of the root cells of other plants. In future studies, we need to buffer solutions of gallic acid in the bioassay experiments, given that the effect of pH on the toxicity of phenolic acids has been well-established (Staman et al. 2001; Blum and Gerig 2005).

In summary, overall, the step-wise experiments performed and the correlative results reported in the present study indicate that *P. australis* secretes a phenolic compound gallic acid into the rhizosphere. The presence of gallic acid in the soil of coastal *Phragmites* marshes has been confirmed. The rhizo-secreted gallic acid produces an allelopathic effect on the tested plants both *in vitro* and in pot experiments under greenhouse conditions. Furthermore, gallic acid inhibited plant growth through ROS-mediated cell death associated with the destruction of the root microtubule assembly.

Acknowledgements HPB acknowledges the University of Delaware and EPSCoR for a faculty start-up grant. The authors thank Dr. Jung-Youn Lee and Dr. Gili Ben-Nissan, Delaware Biotechnology Institute for providing the microtubule-specific GFP-fusion line of Arabidopsis. The authors also thank Dr. Kirk Czymmek and the faculty of the Bio-imaging Center, Delaware Biotechnology Institute for the help with the microscopic studies.

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L-3,4-Dihydroxyphenylalnine (L-dopa), identified from Mucuna (*Mucuna pruriens* L.) (Fujii 1994), is exuded from the roots, and causes growth inhibition in other species that grow close to the plant. Although L-dopa is the precursor for some alkaloids, lignin, phenylpropanoids, and melanin in plant species, excess exogenous L-dopa suppresses root elongation of some plants (Fujii 1994; Nakajima et al. 1999; Hachinohe et al. 2004). In the melanin synthesis pathway, L-dopa is metabolized to dopaquinone by polyphenol oxidase (PPO, EC 1.10. 3. 1, EC 1.14. 18. 1), and then dopaquinone is transformed into brown-colored melanin and melanin-like compounds via several steps that include oxidation and polymerization (Albrecht and Kohlenbach 1990). The melanin synthesis pathway generates reactive oxygen species (ROS), such as O_2^-, $^1O_2$, H_2O_2, and $OH^\bullet$, and free radical species (FRS; Kruk et al. 1999). Although L-dopa is the effective therapeutic agent for Parkinson's disease, it has been reported that the long-term administration of L-dopa induces cell apoptosis and cytotoxicity (Basma et al. 1995; Lai and Yu 1997; Haque et al. 2003). The neurocytotoxicity of L-dopa is considered to be a result of oxidative damage caused by ROS and/or FRS generated from the melanin synthesis pathway (Graham 1978; Basma et al. 1995; Lai and Yu 1997; Haque et al. 2003).

In suspension-cultured carrot cells, we revealed previously that the main factor of the phytotoxicity of L-dopa was oxidative damage by ROS and/or FRS (Hachinohe and Matsumoto 2005). In L-dopa-treated carrot cells, an accumulation of melanin and an increase of lipid peroxide levels occurred. The growth of the cells was restored by a reduction of the amount of melanin and lipid peroxide by application of antioxidants, ascorbic acid and α-tocopherol. Early studies on the phytotoxicity of L-dopa showed that its action was species-selective (Fujii 1994; Nakajima et al. 1999). We have surveyed the effect of L-dopa on 32 species and found tolerant and susceptible species (Hachinohe et al. 2004). However, the mechanism of the species-selective phytotoxicity of L-dopa remains obscure.

The aim of this study was to elucidate the mechanism of species-selective phytotoxicity of L-dopa. The involvement of ROS/FRS in the phytotoxicity of L-dopa was examined in barnyardgrass (*Echinochloa crus-galli* L.) and lettuce (*Lactuca sativa* L.), tolerant and susceptible species, respectively. Additionally, the activity of PPO, a key enzyme of the melanin synthesis pathway, was compared between the species.

Methods and Materials

Growth Conditions and Bioassay Seeds were sown on an aluminum mesh tray covered with cheesecloth and set in a plastic box (310×220×50 mm) that contained distilled water. The water level was adjusted to keep seeds wetted until germination. They were kept in a growth chamber under 25:20°C, 12:12 h L/D conditions. Germinated seeds of 1-day-old lettuce and 4-day-old barnyardgrass were used for bioassays. Ten uniformly germinated seeds of each species were set in a plastic box (60×60×100 mm) that held 200 ml of 0.5% agar culture medium (pH 6.0) containing L-dopa. In addition, ascorbic acid or α-tocopherol was added to the medium to determine the recovery effects of the antioxidants from phytotoxicity of L-dopa on lettuce. Plants were kept in the growth chamber under 25:20°C, 12:12 h L/D conditions for 5 days, and their root lengths were measured with a ruler. Each treatment was conducted with three boxes, and the experiment was repeated three times.

Determination of Lipid Peroxidation Lipid peroxide was determined with the thiobarbituric acid (TBA) test (Velikova et al. 2000). Plant root tissue (500 mg) was homogenized in a

Table 1 Effect of L-dopa on the root growth of barnyardgrass and lettuce

Plants	Root Length (% of Control)				
	10^{-7} M	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3}M
Barnyardgrass	103.7 (8.77)	88.4 (4.90)	89.7 (0.60)	98.2 (18.79)	12.2 (1.12)*
Lettuce	99.0 (3.00)	98.9 (2.20)	29.1 (3.28)*	19.8 (0.35)*	8.9 (0.49)*

Root lengths of untreated control in lettuce and barnyardgrass are 52.5±0.9 mm and 40.7±2.1 mm, respectively. Numbers in parentheses are the ±SE of the mean (N=5).

*P<0.01, a significant preference between control and L-dopa treatment

0.1% (*w*/*v*) trichloroacetic acid (TCA) solution (5 ml) with a pestle and a mortar. The homogenate was centrifuged at 10,000×*g* for 20 min, and 0.5 ml of the supernatant was added to 1 ml of 0.5% (*w*/*v*) TBA in a 20% TCA solution. After the mixture was incubated for 30 min at 98°C, the reaction was stopped by cooling on ice. The sample was centrifuged at 10,000×*g* for 5 min, and the absorbance of the supernatant was measured at 532 nm. The value for non-specific absorbance at 600 nm was subtracted. The amount of thiobarbituric acid reactive substances (TBARS) was calculated with an extinction coefficient of 155 mM^{-1} cm^{-1}.

Spectrophotometric Assay of Melanin Total melanin content was determined at 1, 3, and 5 days after treatment according to Wakamatsu and Ito 2000. Plant root tissue (50 mg) was homogenized in 5 ml of a 90% (*v*/*v*) Soluene-350 solution and solubilized completely at 100°C for 30 min. After cooling, the absorbance at 500 nm was determined with synthetic melanin used as a standard.

Enzyme Extraction and Enzyme Assay A PPO assay was performed according to Vaughn et al. (1988). Root tissue (500 mg) of barnyardgrass and lettuce growing in agar culture medium for 5 days was homogenized with 5 ml of 0.1 M sodium phosphate buffer (pH 7.0) that contained 0.1% Triton X-100. The homogenate was centrifuged at 12,000×*g* for 20 min at 4°C, and the supernatant was used as a crude extract. PPO activity was determined spectrophotometrically by following the conversion of L-dopa to dopaquinone at 490 nm. The reaction medium consisted of 50 mM sodium phosphate buffer (pH 7.0), 100 mM

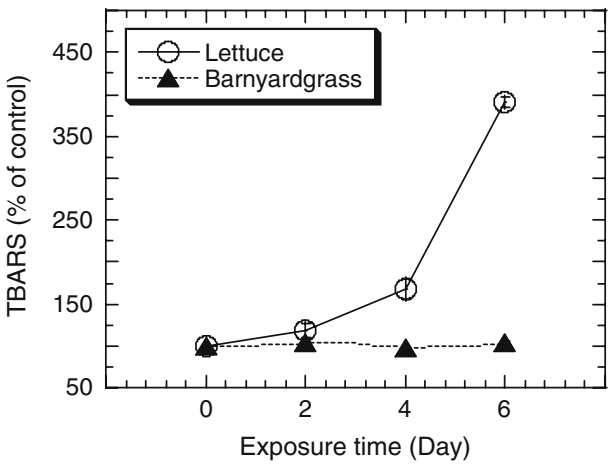


Fig. 1 Effect of L-dopa on lipid peroxide formation in barnyardgrass and lettuce. Concentration of L-dopa was 10^{-4} M. *Bars* indicate ±SE of the mean (N=3)

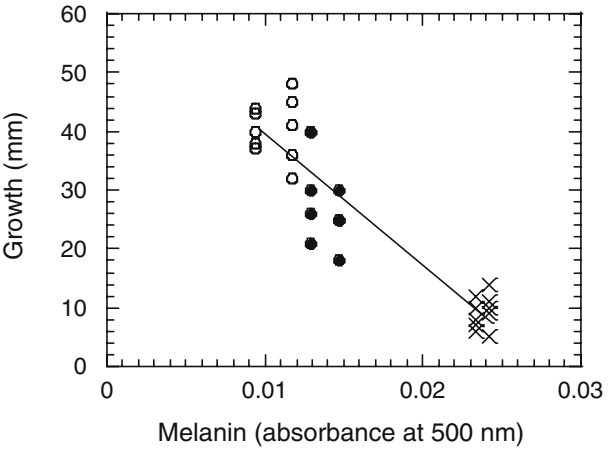


Fig. 2 Relationship between melanin content and elongation in root of lettuce treated with L-dopa for 3 days (r^2=0.85, $F_{1, 28}$=152.8, P<0.001). *Open circle* Control, *filled circle* 10^{-5} M L-dopa, *ex sign* 10^{-4} M L-dopa

L-dopa, 0.15% SDS, and 2,800 U/ml catalase. All assays were conducted in triplicate, and experiments were repeated at least three times.

Statistical Analysis Data were analyzed with Statcel (OMS, Saitama, Japan) and tested for normality and homogeneity of variances (F test or Bartlett test) followed by ANOVA or simple linear regression analysis. Differences between treatments were determined by Fisher's PLSD test, Scheffé's test, Student's t test, or Welch's t test.

Results

The growth suppression by L-dopa differed remarkably between barnyardgrass and lettuce. Root lengths of 10^{-4} M L-dopa-treated plants were approximately 100 and 20% of untreated controls of barnyardgrass and lettuce, respectively (P<0.004, Table 1).

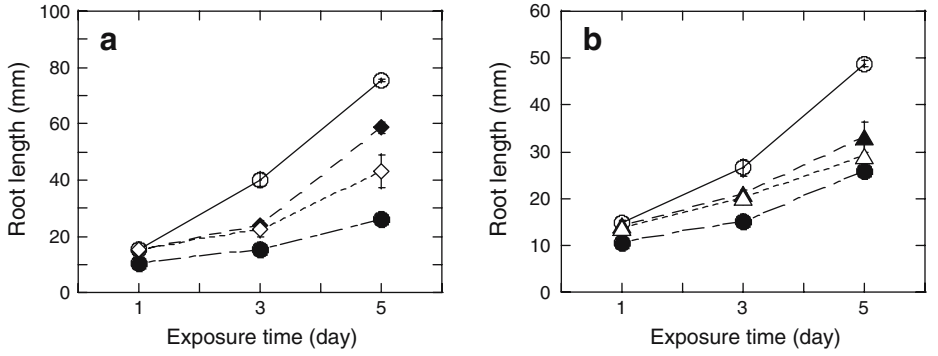


Fig. 3 Effects of ascorbic acid (**a**) and α-tocopherol (**b**) on the growth of lettuce root treated with L-dopa. *Open circle* control, *filled circle* 10^{-5} M L-dopa, *open diamond* 10^{-5} M L-dopa+10^{-8} M ascorbic acid, *filled diamond* 10^{-5} M L-dopa+0.5×10^{-7} M ascorbic acid, open *triangle* 10^{-5} M L-dopa+10^{-7} M α-tocopherol, *filled triangle* 10^{-5} M L-dopa+0.5×10^{-6} M α-tocopherol. A one- way ANOVA was performed: ascorbic acid at 3 days ($F_{3, 8}$=28.47, P<0.001), ascorbic acid at 5 days ($F_{3, 8}$=117.07, P<0.001); α-tocopherol at 3 days ($F_{3, 32}$=35.37, P<0.001), α-tocopherol at 5 days ($F_{3, 32}$=36.60, P<0.001)

Table 2 Effect of antioxidants on the formation of lipid peroxide and melanin in root of lettuce treated with 10^{-5} M L-dopa

Treatment	TBARS (nmol/g F.W.)	Melanin (μmol/mg F.W.)
Control	1.35 (0.10)a	24.0 (0.88)a
Dopa	2.43 (0.20)b	26.4 (0.57)b
Dopa + ascorbic acid	2.06 (0.01)ab	21.4 (0.40)ab
Dopa + α-tocopherol	1.95 (0.06)ab	20.8 (0.16)a

Concentrations of ascorbic acid and α-tocopherol were 0.5×10^{-7} M and 0.5×10^{-6} M, respectively. Numbers in parentheses are the ±SE of the mean (N=3). A one-way ANOVA was performed: TBARS ($F_{4,\ 10}$=137.57, P< 0.001), melanin ($F_{4,\ 10}$=49.37, P<0.001). Means followed different letters differ significantly at P=0.05

The amount of lipid peroxide in L-dopa-treated root of barnyardgrass and lettuce was determined (Fig. 1). The increase of TBARS induced by L-dopa was remarkable in lettuce, reaching 3.8-fold of non-treated control, but little increase was detected in barnyardgrass throughout the exposure period. Regression analysis showed that the content of lipid peroxide in plant roots correlated with the degree of growth inhibition (r^2=0.67, $F_{1,\ 10}$=20.4, P<0.05; data not shown).

Treatment with 10^{-4} M L-dopa for 5 days resulted in greater melanin content, 104 and 270% of non-treated controls in barnyardgrass and lettuce, respectively (data not shown). The amount of melanin accumulated in lettuce root correlated with the growth inhibitory activity of L-dopa (Fig. 2).

The antioxidants ascorbic acid and α-tocopherol reduced the growth inhibition of lettuce roots caused by L-dopa (Fig. 3). Ascorbic acid was more effective than α-tocopherol in suppressing phytotoxicity. Root lengths of lettuce seedlings were rescued up to 80% of the untreated controls when 0.5×10^{-7} M ascorbic acid was included with 10^{-5} M L-dopa. Moreover, melanin accumulation and lipid peroxide formation returned to the same levels as the control in the antioxidant-treated lettuce (Table 2). Catalase and superoxide dismutase (SOD) were not as effective as ascorbic acid or α-tocopherol (data not shown).

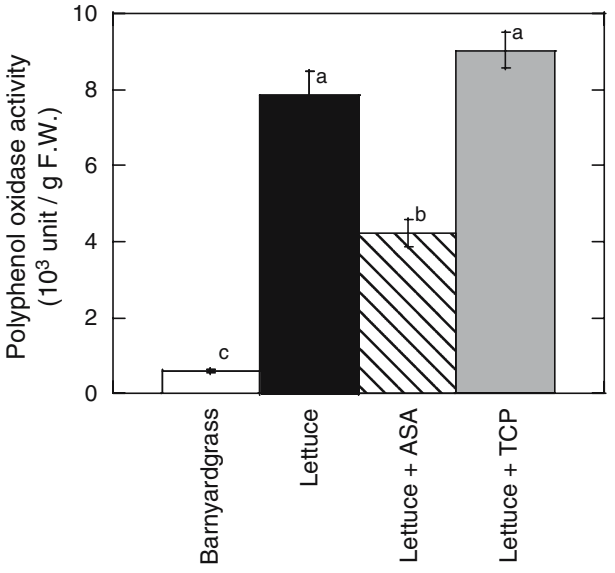


Fig. 4 Activity of polyphenol oxidase in roots of barnyardgrass and lettuce, and the effects of antioxidants on the enzyme activity of lettuce. Concentrations of ascorbic acid (*ASA*) and α-tocopherol (*TCP*) were 0.5×10^{-7} M and 0.5×10^{-6} M, respectively. *Bars* indicate±SE of the mean (N=3). A one- way ANOVA was performed ($F_{3,\ 8}$= 79.64, P<0.001). *Bars* labeled with *different letters* are significantly different (P=0.05)

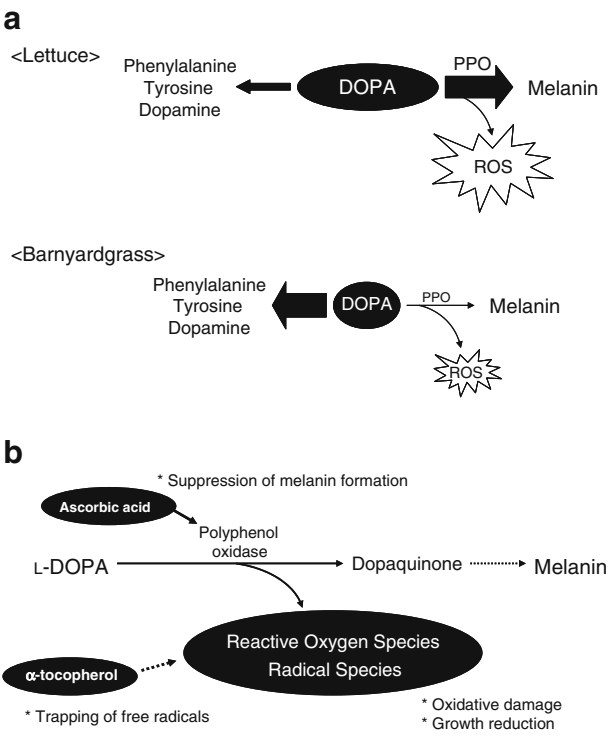


Fig. 5 Proposed mechanism of phytotoxic action (**a**) and selectivity (**b**) of L-dopa

A significant difference in oxidation of L-dopa by PPO between barnyardgrass and lettuce was detected in the roots, with the activity being much greater in lettuce (Fig. 4). The antioxidant effect on PPO activity differed; ascorbic acid suppressed PPO activity in lettuce root extracts but α-tocopherol did not.

Discussion

ROS/FRS cause oxidative damage to membrane lipids and, probably, result in growth inhibition or cell death. Previous work on L-dopa action revealed ROS/FRS involvement in its toxicity on cultured animal cells (Graham 1978; Basma et al. 1995; Lai and Yu 1997; Haque et al. 2003). ROS/FRS are also involved in the phytotoxic action of L-dopa in carrot cells (Hachinohe and Matsumoto 2005). The greater increase of lipid peroxide detected in the roots of lettuce compared to barnyardgrass suggests that the amount of ROS/FRS generated by L-dopa is greater in lettuce, and that the species-selective phytotoxicity of L-dopa between barnyardgrass and lettuce may be attributed to the difference in ROS/FRS generation.

L-Dopa is metabolized to catecholamine, phenylpropanoids, and melanin in plant cells (Pattison et al. 2002). Metabolism to nontoxic amino acids such as phenylalanine or tyrosine is considered to be the detoxification pathway (Nakajima et al. 1999). We previously reported that L-dopa metabolism to phenylalanine or tyrosine is higher in barnyardgrass than in lettuce (Hachinohe et al. 2004). The greater melanin accumulation observed in the roots of lettuce indicates that there is a difference in the activity of L-dopa metabolism in the two species. This difference might account for the species-selective

phytotoxicity. Regression analysis showed that the amount of accumulated melanin correlated with the L-dopa growth inhibitory effect, and melanin synthesis is considered to play a role in L-dopa phytotoxicity.

There are several reports that indicate that the cytotoxicity of L-dopa is reduced by catalase, SOD, ascorbic acid, and α-tocopherol (Parson 1985; Basma et al. 1995; Haque et al. 2003). Ascorbic acid or α-tocopherol both restored the growth of carrot cells treated with L-dopa (Hachinohe and Matsumoto 2005). In this study, ascorbic acid and α-tocopherol also reduced L-dopa phytotoxicity and prevented the formation of melanin and lipid peroxides in the susceptible lettuce species. From the results, we presume that the phytotoxic mechanism of L-dopa on lettuce is a result of oxidative damage by ROS/FRS generated from the conversion to melanin (melanin synthesis pathway). Excess ascorbic acid, however, caused stronger growth suppression and accelerated the formation of lipid peroxide (data not shown). This adverse effect could be caused by the formation of ascorbate radicals that function as oxidants. Antioxidative enzymes, catalase and SOD, did not reduce phytotoxicity, possibly because these macromolecules may not have been taken up through the cell membranes (Buckley et al. 1987; Beckman et al. 1988).

PPO, a copper protein widely distributed in the terrestrial plant species (Sherman et al. 1991), is a key enzyme that regulates the melanin synthesis pathway because it catalyzes a reaction converting L-dopa to dopaquinone (Pattison et al. 2002). Our study showed that PPO activity was significantly higher in lettuce than in barnyardgrass, suggesting that the melanin synthesis pathway is more active, with greater amounts of ROS/FRS and melanin generated. Thus, the difference in PPO activity between the two species is likely to be one of the factors conferring the species-selective phytotoxicity. The antioxidants, ascorbic acid and α-tocopherol, had different effects on lettuce PPO activity. Ascorbic acid may inhibit the melanin synthesis pathway through direct suppression of PPO activity, leading to the reduction of ROS/FRS formation and phytotoxicity. α-Tocopherol, in contrast, is thought to scavenge the ROS/FRS directly, so that while oxidative damage of L-dopa decreased, it caused little effect on PPO activity.

We propose a general mechanism for the phytotoxicity of L-dopa in plant roots, as well as an explanation for its selectivity (Fig. 5). Phytotoxicity of L-dopa in plant roots is due to oxidative damage caused by ROS/FRS generated from the melanin synthesis pathway. The mechanism of species-selective phytotoxicity is likely because of differential PPO activity (Fig. 5a). Furthermore, the mechanism for the alleviation of phytotoxicity might be different between ascorbic acid and α-tocopherol (Fig. 5b).

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Introduction

Males of most moth species locate mates by following a plume of sex pheromone upwind to a calling female. In wind tunnel studies, the structure of the pheromone plume is important in mediating how the responding insect orients during upwind flight to the pheromone source (Mafra-Neto and Cardé 1994, 1995; Vickers and Baker 1994). In the field, pheromone is carried downwind in turbulent eddies, which range in size from centimeters to several meters (Murlis and Jones 1981; Murlis et al. 1992). Most male moths that orient to a pheromone source in a wind tunnel require an intermittent chemical signal that mimics turbulent odor plumes in nature to orient and fly upwind (Kennedy et al. 1980; Willis and Baker 1983; Baker et al. 1985). A combination of self-steered counterturning and odor-triggered upwind optomotor anemotaxis, which is induced by the plume structure, is thought to control a moth's upwind trajectory to the pheromone source (Mafra-Neto and Cardé 1994). The integration of these two mechanisms is modulated "moment-to-moment" by the interception of individual odor pulses comprising the fine-scale structure of turbulent plumes (Mafra-Neto and Cardé 1994, 1995, 1996; Vickers and Baker 1994). Male almond moths, *Cadra cautella*, fly faster and straighter to more turbulent odor plumes than to continuous narrow "ribbon" plumes and also locate the sources of turbulent plumes more frequently than they do ribbon plumes (Mafra-Neto and Cardé 1994). However, males of *C. cautella* are also capable of orienting upwind in a homogeneous cloud of pheromone (Justus and Cardé 2002).

Until recently, the release of chemical stimuli in wind tunnel experiments on insect flight behavior relied mainly on the passive evaporation of volatile chemicals from a lure, usually made from a filter paper or rubber septum. Although the dose applied to these lures is specified, the rate of release is influenced by many factors such as vapor pressure, chemical affinity to the substrate used, the kind and amount of solvent used in application, temperature, airflow over the dispenser, and time. It can be difficult or impossible at very low doses to establish the ratio and emission rates of chemicals released from such traditional lures.

A piezo-electric sprayer system has been developed as a solution to many of the problems associated with traditional lures. The piezo-sprayer system allows the precise release of chemical stimuli as an aerosol (El-Sayed et al. 1999b). The system consists of a drawn glass capillary tip, with an opening of 10–50 μm i.d., which is vibrated at ultrasonic frequency (~120 or ~300 kHz) by a piezo-ceramic disk (El-Sayed et al. 1999b; Skals et al. 2003). This disperses the solution in micro-droplets, producing an aerosol, which evaporates completely within 5 cm of the tip. This system provides benefits over passive release systems because by controlling the exact composition of the pheromone solution used, the release rate of each component within the solution can be defined.

The plume structure generated by these piezo-electric sprayer systems has not previously been documented, and little is known about how the plume structure from such systems is likely to affect flight orientation behavior of insects that orient to the odor plume. The physical configuration of the piezo-sprayer is likely to have large and varied effects on plume structure. To our knowledge, there have been 16 published studies that have used this piezo-sprayer system, 14 laboratory studies (El-Sayed et al. 1999a, b, 2001; El-Sayed 2004; Pettersson et al. 2001; Plepys et al. 2002; Coracini et al. 2003; Skals et al. 2003; Svensson et al. 2004; Trimble et al. 2004; Olsson et al. 2005a, b; Tasin et al. 2006a, b) and two field studies (El-Sayed and Byers 2000; El-Sayed and Trimble 2002). Both field studies gave a clear description of the physical configuration of the piezo-sprayer. Of the 14 laboratory studies, five provided descriptions of the physical configuration of the piezo-

sprayer (El-Sayed et al. 1999b, 2001; Pettersson et al. 2001; Plepys et al. 2002; Skals et al. 2003), and the remaining studies cited previous papers, but provided no indication as to whether the piezo-sprayer configuration was the same as in the papers cited. Four of the papers describe housing the piezo-sprayer inside a cylinder and that uses a distal screen/mesh over the downwind end of the cylinder (El-Sayed et al. 1999b, 2001; Plepys et al. 2002; Skals et al. 2003), but only two of these give measurements of the cylinder diameter and length (El-Sayed et al. 1999b, 2001). None describe the size or shape of the openings in the mesh or their density. All of these factors are likely to affect the plume structure of the odor plume released by the piezo-sprayer, which, in turn, could have effects on orientation of insects flying to the source.

In this study, we used a fast response miniature photoionization (miniPID) detector to perform detailed analyses of the fine-scale structure of a plume generated by a piezo-electric sprayer set up in one of four different physical configurations of the piezo-sprayer in a wind tunnel. Furthermore, we investigated the upwind flight behavior of male *C. cautella* to female sex pheromone released at different concentrations from two different physical configurations of the piezo-sprayer.

Methods and Materials

Wind Tunnel The wind tunnel used was typical of many used to study insect orientation to odors and is described by Justus et al. (2002a). Briefly, it was constructed from 3-mm-thick clear polyacrylic Lexan® bent into an arch-shape and connected to a 1-m-wide frame to create a symmetrical semi-cylinder 3 m in length with a center height of 1 m. The floor was constructed from 10-mm-thick clear Plexiglas® sheets. The wind speed was set to 50 cm/s by regulating two variable output fans, one on the input and one on the exhaust. Air was exhausted out of the building from a semicircular-shaped portion of the tunnel, with a straight base, straight sides, and an arched top, measuring 60 cm wide at the base by 60 cm high at the center of the arch and 30 cm high at the sides before the arch curved, which was located in the center at the base of the tunnel. The remaining air was recirculated into the room. $TiCl_4$ smoke was used to visualize the plume from the piezo-sprayer to ensure a large enough area of the tunnel was exhausted so that all of the pheromone-contaminated air was removed from the room. A 150-mm-thick aluminum honeycomb (Hexcel®, 15-mm diameter cells) was used downwind of the upwind fan to reduce turbulence in the tunnel. On one side of the tunnel, there were two oblong openings, 270 mm from the upwind and downwind ends, to allow access. Openings were covered with clear polyvinyl sheets so that airflow was not disturbed during recordings. The tunnel was housed in a room with a controlled environment of 26–28°C and 50–80% R.H.

Sprayer for Release of Ethanol and Pheromone A piezo-electric sprayer system, described by El-Sayed et al. (1999b), was used to produce an aerosol from both pure ethanol for plume detection and analysis experiments and synthetic pheromone diluted in ethanol for moth flight bioassays. The system consisted of a drawn glass capillary tip, with an opening of 10–50 μm i.d., which was vibrated at ultrasonic frequency by a piezo-ceramic disk. This created an aerosol that evaporated completely within 5 cm downwind of the tip. A disk producing a frequency of about 120 kHz was used for recordings of plume structure. A motor-driven syringe pump (CMA/Microdialysis, Carnegie Medicine AB, Stockholm, Sweden) was used to deliver the odorant solution via 0.12-mm i.d. tubing to the capillary tip at a rate of 10 μl/min. This allowed precise known release rates and ratios to be calculated.

In trial behavioral experiments, moths responded negatively (usually by failing to orient along the plume) to the frequency of sounds emanating from this 120-kHz piezo-ceramic disk. Pyralid moths are sensitive to ultrasound within this frequency range, and some species produce and respond to ultrasound within this frequency range during mate orientation and courtship (e.g., Spangler et al. 1984; Trematerra and Pavan 1995). Therefore, a modification to the piezo-sprayer, described by Skals et al. (2003), was used for behavioral experiments. The modified system used a smaller, thicker piezo-ceramic disk, which produced a frequency of about 300 kHz, beyond the hearing range for pyralids (see Connor 1999 for review). Trials indicated that the plume structure did not vary between the two types of piezo disk. We confirmed that the 300-kHz piezo-ceramic disk did not produce ultrasound within the hearing range of moths, using an UltraSoundGate 116 USB hard disk recording system, in combination with an externally polarized Ultra-SoundGate CM16 condenser microphone with a CMPA preamplifier module (Avisoft Bioacoustics, Berlin, Germany), which has a frequency range of 10–180 kHz.

For experiments of plume structure analysis, four different piezo-sprayer configurations were used: (1) sprayer alone; (2) sprayer encased in the center of an 80-mm diameter by 120-mm-long cylinder made of 1-mm-thick polyethylene, with the capillary tip positioned 50 mm from the downwind end of the cylinder, and the downwind end covered by either (a) a 1.6-mm steel mesh, or (b) a 3.2-mm steel mesh, or (c) a 5-cm circular baffle, 70 mm upwind of the sprayer tip. For all configurations, the sprayer was mounted on a stand consisting of a square Plexiglas® base plate, 60×60×4 mm, which had a hole (6.4-mm diameter) drilled in the center of the square. A Teflon® tube, 80 mm in length by 6.4 mm in diameter was inserted into the hole in the base plate, and an 83-mm-high black plastic holder, to mount the capillary tip and the piezo disk, was inserted into the top end of the Teflon® tube. This resulted in the capillary tip being mounted 167 mm above the tunnel floor.

Measurement of Air Velocity around the Piezo-Sprayer Air velocity was measured around the four different piezo-electric sprayer configurations with a Barnant Tri-Sense anemometer (Barrington, IL, USA), at the following locations in relation to the piezo disk: (1) 10 cm upwind; (2) 3.5 cm upwind; 3) 10 cm downwind; (4) 5 cm lateral; (5) 10 cm lateral; (6) 5 cm above; and (7) 10 cm above.

Plume Detection and Analysis The plume structures produced by the four piezo-sprayer configurations were measured with a fast-response photoionization detector (miniPID; Aurora Scientific, Aurora, Ontario, Canada) with a frequency response rate of 330 Hz and a detection threshold of 100 ppb propylene. This miniPID sensor is sensitive to any substance with an ionization potential equal to or below approximately 10.6 eV. Ethanol was used to form the aerosol released from the piezo-sprayer (ionization potential 10.6 eV), and it was also the solvent used to dilute the pheromone in behavioral experiments. Hexane, a more commonly used solvent for dilution of pheromones, was not detectable by the miniPID. In addition, in previous experiments with the piezo-sprayer (P. Witzgall, personal communication) and also in behavioral trials for these experiments, hexane inhibited moth orientation upwind.

The sensor was mounted on a computer-controlled traverse, as used in Justus et al. (2002a), with movement in the longitudinal (x), horizontal (y), and vertical (z) axes. This allowed the miniPID to be placed within the wind tunnel with a precision of 0.25 mm. The sampling rate of the miniPID was set at 1,250 cm^3 min^{-1} through an inlet needle with a 0.076-cm internal diameter. The miniPID controller has three gain settings (1, 5, and 25) and a potentiometer for adjusting the baseline signal level. Noise measured from the probe

running in the tunnel without the piezo-electric sprayer was less than 1 mV rms at the lowest gain setting.

For all piezo-sprayer configurations, measurements of plume structure were taken at 100, 200, and 400 mm from the tip of the vibrating glass capillary. This region near the source is of particular importance because it is within this range that a moth appears to alter its flight behavior as it approaches the odor source (Murlis and Bettany 1977; Murlis et al. 1982; Willis and Baker 1994). The output signal from the sensor was recorded for durations of 15 sec at 3-mm intervals, for a 12×12-mm square at the core of the plume, for a total of 25 recordings, which were then averaged to give mean values for the plume core. The center point of the 12×12-mm square was directly in line with the capillary tip of the piezo-sprayer. Additionally, recordings of 15-sec duration were made every 3 mm along the vertical and horizontal axes within the cross-section of the plumes. A duration of 15 sec is standard for these kinds of experiments (Justus et al. 2002a). Blank recordings, where the piezo-sprayer was turned off, were recorded approximately every 20 recordings, to acquire a baseline. The analog voltage signals from the miniPID were digitized and logged on a computer by using a GemAcq-4data acquisition system (Gemini Enterprises, UK). The sampling frequency was set at 100 Hz (this is sufficiently high to resolve structure perceived by a moth's central nervous system, which has been recorded to have a maximum frequency response of the order of 10 Hz (Christensen and Hildebrand 1988; Vickers et al. 2001), and the full voltage span of 5 V was digitized at 12-bit resolution. Data are presented as voltages to give a relative measure of concentration, which is comparable between recordings. In subsequent statistical analyses (see below), a discrimination threshold of 0.014 mV rms (approximately four times the root mean square noise determined by inspection of recordings) was used to reject noise.

Statistical Analyses of Plume Structure Statistics were calculated by averaging the 25 recordings made within each 12×12-mm core along each plume's axis at the three distances downwind. The characteristics measured for each 15-sec sample of the sensor signal were as follows: (1) relative conditional mean concentration (conditional averages are those formed from ensembles of data taken when signal is present, for example, see Mylne and Mason 1991); (2) intermittency (defined here as the probability of zero signal, $1-\gamma$, the proportion of time for which concentration was below the noise threshold, following the convention of Murlis and Jones (1981) and Jones (1983); and (3) peak-to-conditional-mean ratio (the ratio of the highest registered value in a sample to the overall conditional mean of the sample; this eliminates the influence of intermittency on the conditional mean). Each of these parameters was tested for normality using a Ryan–Joiner test, and none was found to be normally distributed. As a result, all parameters were log-transformed and tested again to confirm they were normally distributed. Analyses of variance (ANOVAs) were run on these log-transformed parameters for both within plumes at various distances from the source and among plume types. All statistical tests were performed using Minitab v.14. For measures of intermittency, both horizontally and vertically through the plume, trend lines were fitted using a four-parameter sigmoidal curve.

Insects Cadra cautella were reared on a diet of poultry laying mash, rolled oats, brewer's yeast, and glycerin (Mafra-Neto and Cardé 1995). Insects were reared at about 27°C, with a 16L:8D light regime and 50–60% R.H. Last instar larvae were sexed, and males were isolated in plastic cups (163 ml), filled one third full of the above diet, until eclosion. Eclosed males were transferred once daily to 30×30×30-cm plastic screened cages. All individuals used in experiments were between 2- and 4-d-old.

Moth Flights to Sprayer Male *C. cautella* were flown to two of the different piezo-sprayer configurations, the sprayer alone and the sprayer with an upwind baffle. These two configurations were chosen because the sprayer alone was the simplest configuration and because in plume structure analysis, the upwind baffle showed the greatest variation from the sprayer alone in a number of the variables recorded. Synthetic pheromone in the natural 13:1 ratio (Allison and Cardé 2006) of the two major components, (9*Z*,12*E*)-tetradecadienyl acetate (Z9E12-14:Ac) and (*Z*)-9-tetradecenyl acetate (Z9-14:Ac), was formulated in ethanol to produce four concentrations of pheromone, which, when released by the piezo-sprayer at 10 μl/min resulted in release rates of 1, 10, 100, or 1,000 pg/min of Z9E12-14:Ac. On a single day, ten moths were flown to each of the four different release rates, for one of the piezo-sprayer configurations, and this was repeated over 4 d, giving a total of 40 moths being flown to each pheromone concentration for each piezo-sprayer configuration. The same experiment was then repeated over another 4 d for the other piezo-sprayer configuration. It was not possible to conduct experiments for the two different configurations on the same day with the same cohort of moths, because of technical and temporal problems associated with switching between configurations, which is especially complicated when using the approximately 300-kHz piezo system because it is extremely sensitive and time-consuming to set up within the moths 2-hr flight period. The order of release rates being tested on each day was decided by using a Latin square design.

All experiments were conducted in the first 2 hr of the scotophase. A male *C. cautella* was chosen at random and held in a small wire cage with one open face kept covered in the odor plume at the downwind end of the tunnel. When the male initiated wing fanning, the open face was uncovered, and the male was allowed to fly upwind. For each male flown, the following behaviors were recorded: (1) activation (wing fanning and walking in the wire cage); (2) taking flight; 3) upwind flight; (4) oriented flight (upwind flight to within 30 cm of the piezo-sprayer); and (5) landing at the source. The landing substrate was larger for the upwind baffle configuration, given that it included the 5 cm circular baffle upwind of the source. Additionally, the time taken from takeoff to reach the source for successful flights was recorded. In experiments with the upwind baffle configuration, the baffle was changed between each change of pheromone concentration.

The tunnel floor was covered with white fabric, and 5.4-cm-diameter red dots were randomly distributed but evenly spaced at 30 circles per square meter to provide non-directional visual cues. All experiments were conducted at about 26°C. Two lateral banks of 25 W, clear red lights (Philips Colortone®) on each side of the room illuminated the ceiling and provided a diffuse light of 7–10 lx in the wind tunnel. Two Sanyo VCB-3512T monochrome CCD cameras (shutter speed 1/100 sec) were mounted around the wind tunnel; one at the side (with a 75-mm lens) and one above the wind tunnel (with a 4.8-mm lens), to record the moths' flight and behavioral responses.

For both configurations tested, responses within each of the behavioral parameters to each of the four pheromone concentrations for each of the 4 d of testing were first subjected to a heterogeneity *G* test. If not significant, they were then pooled and compared using a series of pooled *G* tests for goodness of fit (Sokal and Rohlf 1995). The time taken to reach the source was tested for normality using a pair of Shapiro–Wilk tests for the two piezo-sprayer configurations and then compared with a pair of ANOVAs. Statistical tests were performed using SPSS v. 15. It was not appropriate to make statistical comparisons of moth behavior between the two piezo-sprayer configurations because tests of different configurations could not be performed on the same day with the same cohort of moths.

Results

Air Velocity around Piezo-Sprayer The presence of the sprayer alone in the wind tunnel reduced the 50 cm/sec air velocity in the wind tunnel (Table 1). Relative to the measurements made around the sprayer alone, air velocities around the other piezo-sprayer configurations were higher at 10 cm upwind and at both 5 and 10 cm both laterally and dorsally of the piezo disk, but were lower at 3.5 cm upwind of the piezo disk and 10 cm downwind of the piezo disk. Furthermore, from observing the data, air velocities appeared to be reduced more for the upwind baffle configuration at 3.5 cm upwind and 10 cm downwind, and air velocity also appeared to increase more for the upwind baffle at 5 cm dorsally than for the two mesh configurations.

Plume Structures of the Different Piezo-Sprayer Configurations Representative traces of typical relative ethanol concentrations over a 15-sec period at the core of the plume are shown in Figs. 1, 2, 3, 4. There was discernible variation among the different piezo-sprayer configuration in both the amplitude and periodicity of the signals. In general, concentrations declined as the distance from the piezo-sprayer increased; however, for the coarse mesh sprayer configuration, the concentration of the signal at 200 mm from the source was greater than at 100 mm before decreasing again by 400 mm from the source (Fig. 3). The relative ethanol concentrations decreased at different rates with different configurations likely because of variations in the plume size from each configuration as the plume was carried downwind.

In measurements of the 12×12-mm plume core, the conditional mean concentrations varied between piezo-sprayer configurations at 100 mm (ANOVA, $F_{3,91}=315$, $P<0.001$), 200 mm (ANOVA, $F_{3,96}=178$, $P<0.001$), and 400 mm (ANOVA, $F_{3,96}=520$, $P<0.001$) downwind from the source. Furthermore, conditional mean concentrations also varied among the different distances downwind of the source for each of the individual piezo-sprayer configurations: sprayer alone (ANOVA, $F_{2,72}=37.8$, $P<0.001$); fine mesh (ANOVA, $F_{2,72}=358$, $P<0.001$); coarse mesh (ANOVA, $F_{2,67}=9.04$, $P<0.001$); and upwind baffle (ANOVA, $F_{2,72}=654$, $P<0.001$; Fig. 5a).

Table 1 Air velocity recordings measured using an anemometer, around a piezo-electric sprayer alone in a wind tunnel, and air velocity measurements from around three other piezo-sprayer configurations, relative to the air velocities recorded when the sprayer alone was in the tunnel

Distance from Piezo-Electric Sprayer	Piezo-Electric Sprayer Configurations			
	Sprayer Alone	Cylinder and Fine Mesh	Cylinder and Coarse Mesh	Upwind Baffle
	Air Velocity (cm/sec)	Air Velocity Relative to the Velocities Measured for the Sprayer Alone (cm/sec)		
10 cm upwind	49	+6	+5	+7
3.5 cm upwind	42	−9	−3	−29
10 cm downwind	40	−13	−7	−26
5 cm lateral	40	+13	+10	+14
10 cm lateral	40	+12	+10	+11
5 cm dorsal	42	0	+4	+9
10 cm dorsal	42	+3	+3	+4

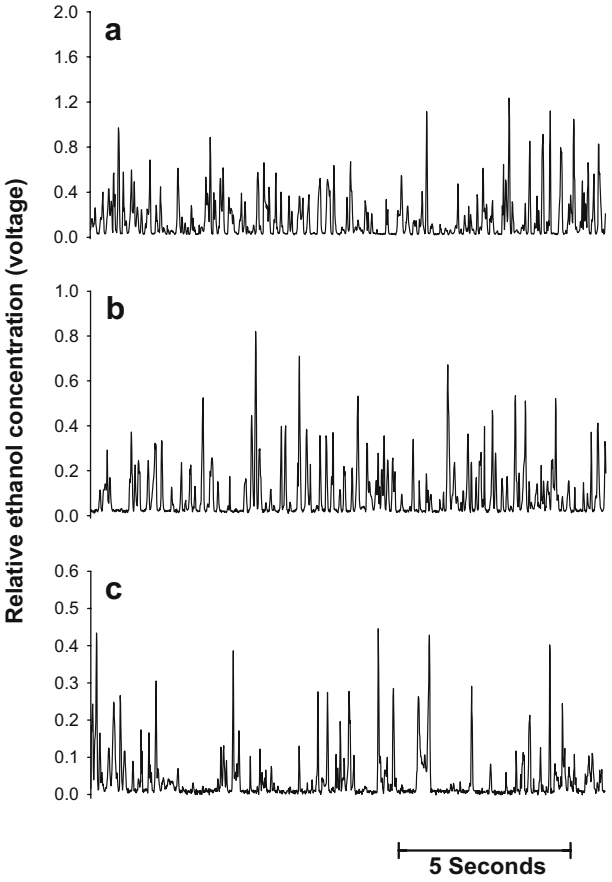


Fig. 1 Representative traces of ethanol concentration at the center of the plume of the piezo-electric sprayer alone, collected using a photoionization detector at three distances downwind of the plume source of the piezo-electric sprayer, in a wind-tunnel: **a** 100 mm; **b** 200 mm; and **c** 400 mm. Each trace represents 15 sec

The fine mesh configuration had significantly higher conditional means than all other piezo-sprayer configurations at both 100 and 200 mm downwind of the plume source (Fig. 5a). These differences in conditional means may be explained by the fact that the fine mesh configuration greatly reduced the air velocity through the cylinder, but increased the airflow around the edges of the cylinder (Table 1), suggesting a pocket of slower moving air in the downwind wake where ethanol concentrations could have built up. This would also explain the fact that at 400 mm downwind, the conditional mean concentrations in the air, which had more time for mixing, were closer to the conditional means of the other piezo-sprayer configurations. The upwind baffle produced similar air velocity changes to the fine mesh (Table 1), but the conditional mean concentrations for these two piezo-sprayer configurations were dissimilar (Fig. 5a). This may be because the diameter of the upwind baffle (50 mm) was smaller than that of the mesh covered cylinder (80 mm) and the baffle was further away from the miniPID detector than the mesh of the cylinder. Therefore, at all measurement points downwind of the source, there was a greater distance for mixing of the air plume behind the upwind baffle configuration than behind the fine mesh configuration.

The coarse mesh configuration had a lower conditional mean concentration at 100 mm from the plume source than at 200 mm. The 25 individual measurements made at 100 mm, of the 12×12-mm plume core, showed that the measurements in the dorsal region of the

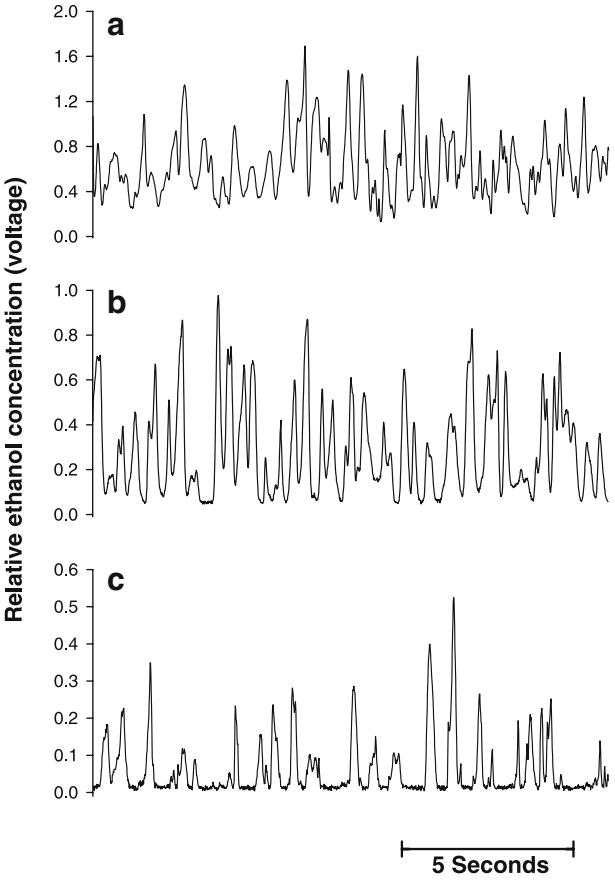


Fig. 2 Representative traces of ethanol concentration at the center of the plume of the fine mesh piezo-electric sprayer configuration, collected using a photoionization detector at three distances downwind of the plume source of the piezo-electric sprayer, in a wind-tunnel: **a** 100 mm; **b** 200 mm; and **c** 400 mm. Each trace represents 15 sec

plume were higher than the measurements in the ventral region of the plume, suggesting that at this distance, the speed of the plume core decreased for this configuration likely because of differences in the air flow around this configuration, which did not occur for the other configurations. Furthermore, lateral and vertical dissections of the plumes showed that the plume for the coarse mesh was narrowest (Fig. 6c and g), and, therefore, the measurements made of this plume were most likely to miss the plume core.

Intermittency at the plume core varied among piezo-sprayer configurations at 100 mm (ANOVA, $F_{3,96}=57.0$ $P<0.001$), 200 mm (ANOVA, $F_{3,96}=38.2$, $P<0.001$), and 400 mm (ANOVA, $F_{3,96}=36.3$, $P<0.001$) downwind from the source. Furthermore, intermittency values also varied among the different distances downwind of the source for each of the individual piezo-sprayer configurations: sprayer alone (ANOVA, $F_{2,72}=110$, $P<0.001$); fine mesh (ANOVA, $F_{2,72}=5040$, $P<0.001$); coarse mesh (ANOVA, $F_{2,72}=3.13$, $P=0.05$); and upwind baffle (ANOVA, $F_{2,72}=265$, $P<0.001$; Fig. 5b). Generally, intermittency values increased with distance from the source for all configurations (Figs. 1, 2, 3, 4), and, therefore, the closer to the source the measurements were made, the less intermittent the plume was. The exception was the coarse mesh configuration where intermittency decreased with distance from the piezo-sprayer. This may be because of the plume dropping and being narrowest for the coarse mesh configuration.

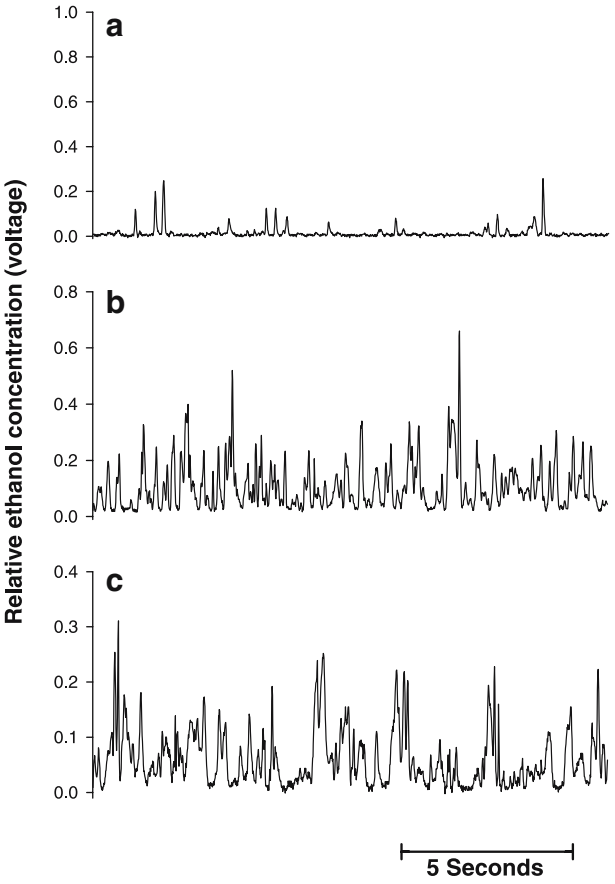


Fig. 3 Representative traces of ethanol concentration at the center of the plume of the coarse mesh piezo-electric sprayer configuration, collected using a photoionization detector at three distances downwind of the plume source of the piezo-electric sprayer, in a wind-tunnel: **a** 100 mm; **b** 200 mm; and **c** 400 mm. Each trace represents 15 sec

Intermittency values at both 100 and 400 mm from the source, for all configurations, from the cores of the plumes to their edges showed a general trend, with intermittency values being low at the plume center and increasing with increasing distance, i.e., the probability of a zero signal increased with distance from the plume center (Fig. 6). Additionally, the width and height of plumes varied greatly between configurations (Fig. 6). In general, at both 100 and 400 mm from the source, the coarse mesh resulted in the narrowest plume (Fig. 6c and g), and the upwind baffle resulted in the broadest plume (Fig. 6d and h).

The ratio of peak to conditional mean concentration gives a measure of signal fluctuation; a larger ratio indicates a greater contrast between the peak amplitude and the conditional mean. The ratio of peak to conditional mean concentration at the plume core varied among piezo-sprayer configurations at 100 mm (ANOVA, $F_{3,89}=57.6$, $P<0.001$), 200 mm (ANOVA, $F_{3,96}=55.6$, $P<0.001$), and 400 mm (ANOVA, $F_{3,96}=55.5$, $P<0.001$) downwind from the source. Furthermore, the ratio of peak to conditional mean concentrations also varied among the different distances downwind of the source for each of the individual configurations: sprayer alone (ANOVA, $F_{2,72}=49.0$, $P<0.001$); fine mesh (ANOVA, $F_{2,72}=43.0$, $P<0.001$); coarse mesh (ANOVA, $F_{2,65}=4.86$, $P=0.011$); and upwind baffle (ANOVA, $F_{2,72}=15.30$, $P<0.001$; Fig. 5c). These data indicate that for the

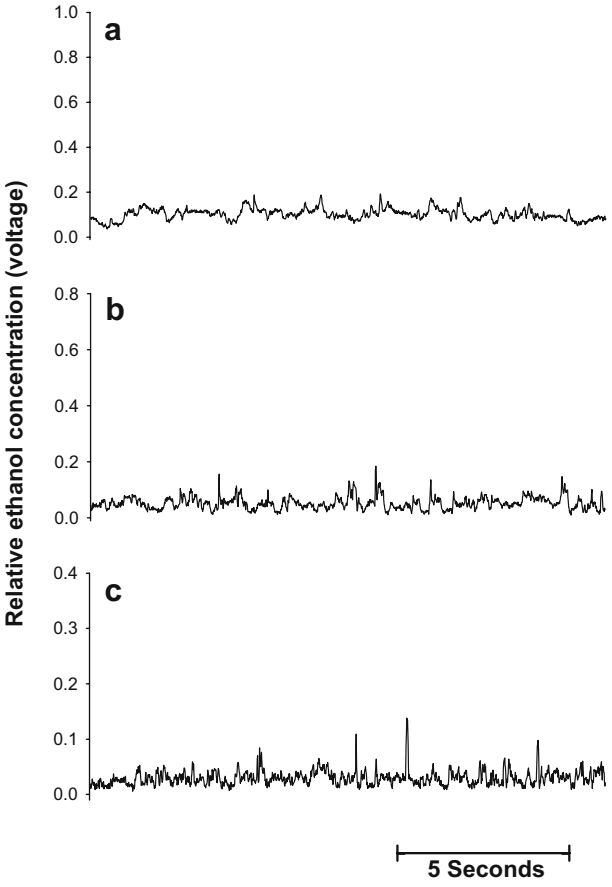


Fig. 4 Representative traces of ethanol concentration at the center of the plume of the upwind baffle piezo-electric sprayer configuration, collected using a photoionization detector at 3 distances downwind of the plume source of the piezo-electric sprayer, in a wind-tunnel: **a** 100 mm; **b** 200 mm; and **c** 400 mm. Each trace represents 15 sec

sprayer alone, coarse mesh, and upwind baffle configurations, signal fluctuation decreased with distance from the source; however, for the fine mesh, the reverse was true. In general, the sprayer alone produced the most fluctuating stimulus intensity in the plume and the upwind baffle the least.

Moth Flights In experiments with both the sprayer alone and with an upwind baffle, high levels of early stage behavioral responses were observed, with nearly all moths tested activating and initiating flight and a large proportion flying upwind along the odor plume (Fig. 7). In the experiments with the sprayer alone configuration, there were no significant differences in the numbers of individuals performing activation, flight, or upwind flight among the different pheromone release rates. However, there were differences in the number of individuals completing oriented flight between the 100 pg/min and 1,000 pg/min release rates (G_1=7.95, P<0.01) and in the number of individuals landing between the 100 pg/min and 1,000 pg/min release rates (G_1=5.82, P<0.05; Fig. 7a). With the 100 pg/min pheromone release rate, more males performed oriented flight and landing than for any of the other release rates. In comparisons between the mean times from takeoff to landing of moths, there were no differences among pheromone release rates (Table 2).

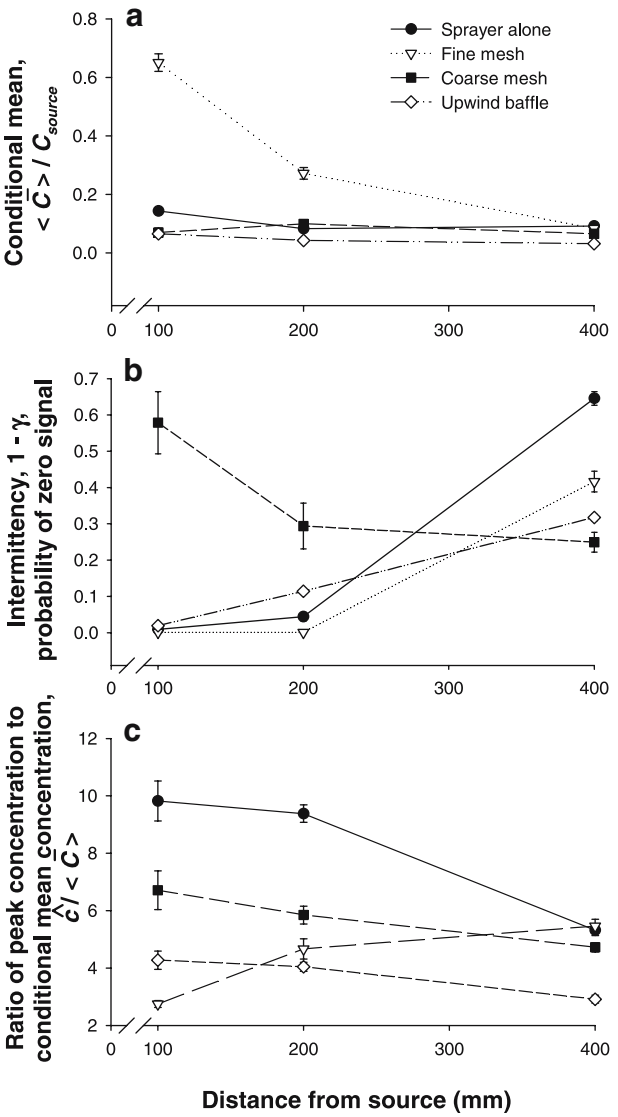


Fig. 5 Mean recordings collected using a photoionization detector from the 12×12-mm core of plumes emitted from four piezoelectric sprayer configurations at three distances downwind from the plume source for: **a** conditional mean concentrations (±SE; N=25); **b** intermittency values, $1-\gamma$ (±SE; N=25); and **c** peak-to-conditional-mean ratios (±SE; N=25)

In experiments with the upwind baffle configuration, there were no differences in the numbers of individuals performing either activation, flight, or upwind flight between different pheromone release rates. However, there were differences in the number of individuals completing oriented flight between the 1 and 1,000 pg/min (G_1=9.66, P<0.01) and the 10 and 1,000 pg/min release rates (G_1=11.28, P<0.001). In addition, there were differences in the number of individuals landing between the 1 and 1000 pg/min (G_1=4.72, P<0.05), the 10 and 1,000 pg/min (G_1=7.95, P<0.01), and the 10 and 100 pg/min release rates (G_1=4.28, P<0.05; Fig. 7b). With the 10 pg/min pheromone release rate, more males performed upwind flight, oriented flight, and landing than for any of the other release rates. In comparisons between the mean times from takeoff to landing of moths, there were no differences between pheromone release rates (Table 2).

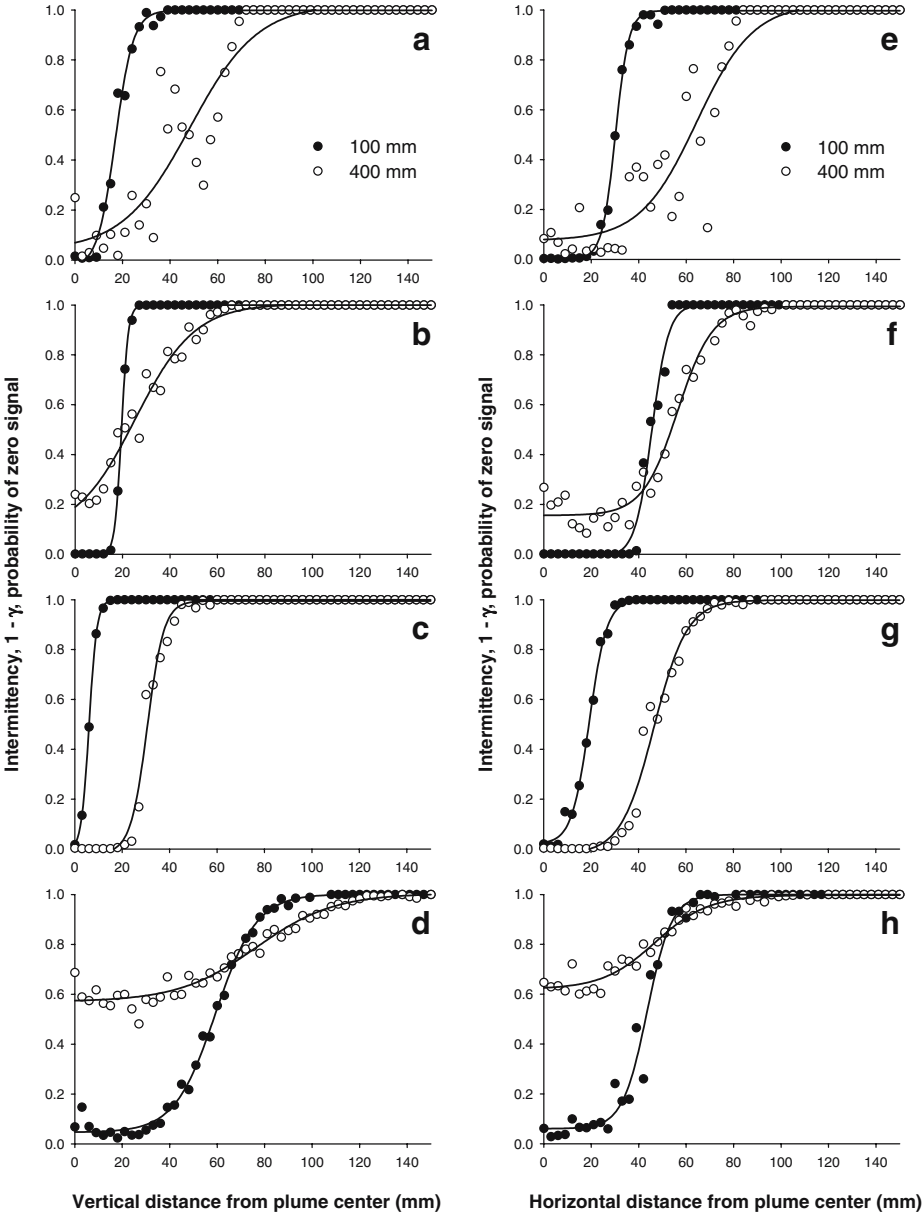


Fig. 6 Intermittency values, $1-\gamma$, recorded using a photoionization detector through four different plumes from the four piezo-electric sprayer configurations, measuring from each plume's axial center moving towards its edge, both vertically upwards through the plume: **a** sprayer alone; **b** fine mesh; **c** coarse mesh; and **d** upwind baffle, and laterally through the plume: **e** sprayer alone; **f** fine mesh; **g** coarse mesh; and **h** upwind baffle (N=25). All trend lines are fitted using a four-parameter sigmoidal curve

Tests for the two configurations could not be conducted on the same day with the same cohorts of moths because of the time taken to set up the apparatus. Therefore, it would not be valid to make statistical comparisons between moths tested with the two different piezo-sprayer configurations. Moreover, the visual cues presented by the two configurations may

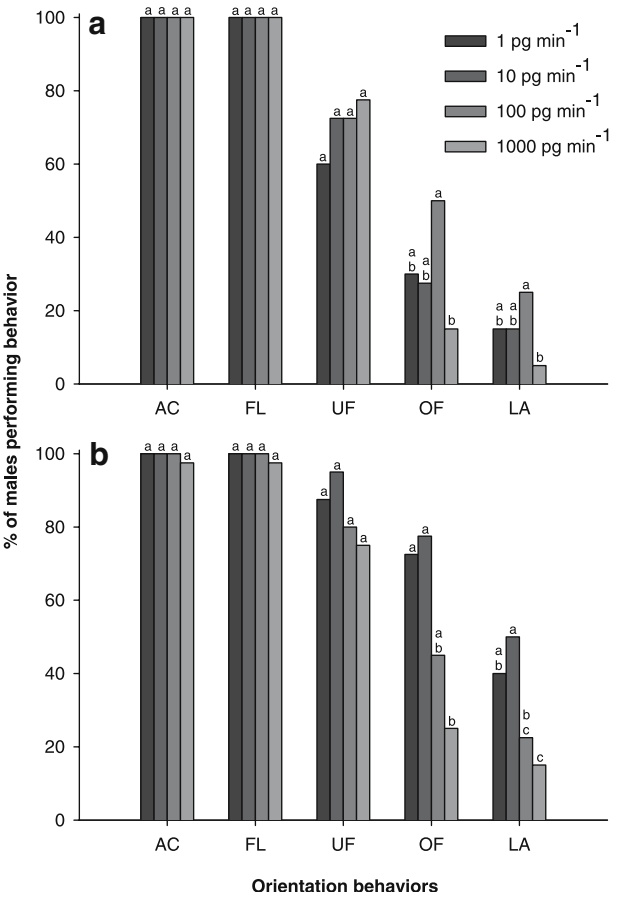


Fig. 7 Flight responses of *C. cautella* to four different release rates of a 13:1 blend of Z9E12-14:Ac and Z9-14:Ac diluted in ethanol from a piezo-electric sprayer system with either: **a** the sprayer alone (N=40) or **b** sprayer with an upwind baffle configuration (N=40). Orientation behaviors are as follows: *AC* Activation, *FL* flight, *UF* upwind flight, *OF* oriented flight, *LA* landing. For each configuration, differences between letters within a behavioral category represent a significant difference at $P<0.05$

have influenced later behaviors, especially propensity to land. Although it was not statistically valid to compare the results between the two sprayers, visual comparisons of the data were made to assess whether any discernable trends could be identified. These comparisons suggested differences among configurations in the level of response and attraction of moths, in close orientation and landing (Fig. 7), in the time taken to reach the source by moths, with moths flying more directly to the upwind baffle (Table 2), and in the level of behavioral responses by moths to the different pheromone release rates, with moths

Table 2 Mean time from takeoff of *Cadra cautella* moths to landing at the source, in seconds (±SE), for two different piezo-electric sprayer configurations, with four different release rates for each configuration

	Piezo-Sprayer Configuration							
	Sprayer Alone				With Upwind Baffle			
Pheromone release rate (pg/min)	1	10	100	1,000	1	10	100	1,000
Mean time from takeoff of moth to landing (sec±SE)	49.5±4.3	51.3±9.3	42.3±7.9	27.0±3.0	20.0±3.1	30.3±6.1	18.1±4.4	23.5±5.1

attracted more strongly to the upwind baffle configuration at lower pheromone concentrations (Fig. 7). The upwind baffle had a significantly lower conditional mean than the sprayer alone at all distances from the piezo-sprayer (Fig. 5a), a broader horizontal and vertical expanse than the sprayer alone (Fig. 6a,d,e, and h), and was significantly more intermittent in the core at 100 and 200 mm from the source, but significantly less intermittent at 400 mm from the source (Fig. 5b).

Discussion

Measurements of the plume structures generated by a piezo-sprayer indicated that the physical configuration of the experiment altered the plume's amplitude and periodicity. At each of the distances downwind from the source, significant differences were found in all of the plume variables among each of the four configurations. Furthermore, in comparisons between results for each of the three distances downwind from the source, separately for each of the different piezo-sprayer configurations, there were significant differences between each of the distances in every comparison made for each of the plume variables measured (Fig. 5), indicating that both configuration and distance from source had significant effects on plume structure. Justus et al. (2002a) demonstrated similar findings in wind tunnel studies in which they compared the effects of changing the configuration of an odor release system, which produced either a stream or a puff of odor from the tip of a glass pipette, resulting in significant changes to all aspects of the plume structure.

Furthermore, our results demonstrated that even minor changes to the configuration of the piezo-sprayer resulted in major changes to the resultant plume structure. For example, with the piezo-sprayer encased in an open-ended cylinder with a distal mesh over the downwind end of the cylinder, changing the distance between the wires in the distal mesh from 1.6 to 3.2 mm resulted in differences between all measures of plume structure (Figs. 2, 3, 5, and 6). This configuration of piezo-sprayer has commonly been used in behavioral experiments (El-Sayed et al. 1999b, 2001; Plepys et al. 2002; Skals et al. 2003); however, in those experiments, the distance between the wires, or any other measures of the structure of the mesh used, were not reported.

In the behavioral experiments, changing the release rate of pheromone had significant effects on the proportion of individuals performing both oriented flight and landing behaviors for both configurations tested (Fig. 7). These results were in agreement with previous studies, which demonstrated that the concentration of pheromone released modulated moth orientation behavior (e.g., Cardé and Hagaman 1979; Keunen and Baker 1982; El-Sayed et al. 1999b). In flight bioassays, more moths landed on the sprayer alone configuration when the pheromone release rate was set to 100 pg/min than to any of the other release rates, whereas for the upwind baffle configuration more moths landed when the release rate was set to 10 pg/min (Fig. 7). Similarly, El-Sayed et al. (1999b) demonstrated that 100 pg/min was the optimal release rate of the major pheromone component for attraction of the grapevine moth, *Lobesia botrana*, when using a piezo-sprayer operating at 120 kHz.

Differences in the fine structure of a pheromone plume have more influence on the flight pattern of *C. cautella* males than a 1,000-fold range in pheromone dose (Mafra-Neto and Cardé 1995). In the present tests, moths responded more strongly to different release rates from the two different sprayer configurations, suggesting that these differences may be caused by differences between the plumes from the different configurations. Furthermore,

from visual comparisons of the data, there appeared to be other differences in behavioral responses of moths between configurations (Fig. 7, Table 2). However, before any conclusions can be drawn about the effects that differences in plume structure can have on flight behavior, these bioassays must be confirmed with fully comparable experiments, where flights to both configurations are tested on the same day with the same cohort of moths, to eliminate any daily bias and any variation in moth response between days.

The levels of behavioral responses in this experiment, specifically landing, were lower than demonstrated in previous experiments with *C. cautella* flying to pheromone applied to a filter paper lure using an almost identical configuration of an upwind baffle (Mafra-Neto and Cardé 1995). In the filter paper lure experiments, a wide range of pheromone concentrations were used, likely to encompass similar concentrations as used in the current experiments. Furthermore, the pheromone components were applied to the filter paper in the same ratio as used in the current tests and, therefore, because of the similar size and volatility of the two pheromone components, it is likely they were released from the filter paper in a similar ratio to which they were applied to the filter paper, making the results of these previous tests comparable to the current experiments. With the filter paper lure, landing percentages ranged from about 70% to about 82% for a variety of pheromone doses in comparison to the 15 to 50% of moths landing for the upwind baffle configuration in the tests reported here. This trend has also been demonstrated in previous studies. For example, males of the codling moth, *Cydia pomonella*, only landed at the source 53±5.7% of the time in piezo-sprayer experiments with a release rate of 50 pg/min of synthetic (*E*,*E*)-8,10-dodecadien-1-ol (codlemone; El-Sayed et al. 1999a) in comparison to about 80% of the time in experiments that use a traditional rubber septum lure, with a release rate of about 40 pg/min (McDonough et al. 1993). However, El-Sayed et al. (1999a) demonstrated an 82±3.4% codling moth landing response to a 100 pg/min release rate. Similarly, landing responses of males of the Brazilian apple leafroller, *Bonagota cranaodes*, were low for both synthetic pheromone blends (ranging from 7–34%) and female gland extracts (57%; Coracini et al. 2003). However, high levels of response to pheromone have been obtained with the piezo-sprayer. For example, the pyralid moth *Plodia interpunctella* exhibited high percentages of source landing to a four-component pheromone blend (Svensson et al. 2004). Similarly, the tortricid *L. botrana* showed frequent (about 80%) landing responses to a three-component pheromone blend (El-Sayed et al. 1999b). It is difficult to compare landing rates or other behaviors in these different trials because plume structures would have varied with the lure type and sprayer configuration.

Because the peak of responses to the piezo-sprayer occurred at 10–100 pg/min, it is likely that the concentrations used in the present experiments were optimal and, therefore, it is unlikely that this was the reason for lower responses to the piezo-sprayer than to traditional lures. The piezo-sprayer used in these experiments used a high frequency of ultrasound (about 300 kHz; Skals et al. 2003) above that to which moths are known to respond (Connor 1999). Furthermore, in previous studies where similar responses were obtained, tortricid moths were used (e.g., Coracini et al. 2003; El-Sayed 2004), which are not known to possess hearing organs. Therefore, it is unlikely that ultrasound was the cause of differences between responses to the piezo-sprayer and traditional lures. Our experiments demonstrate that the plume structures from the piezo-sprayer system were analogous to those produced by more traditional pheromone release systems (Justus et al. 2002a) to which good levels of attraction have previously been recorded (e.g., Justus et al. 2002b).

The lower responses of moths to the piezo-sprayer system may be a result of the solvent released by the piezo-sprayer, which typically uses a flow rate of 10 μl/min. In addition, it has been suggested that ethanol has fewer negative effects on flight behavior than hexane

(P. Witzgall, personal communication), and in our own experiments with the piezo-sprayer, *C. cautella* responded less strongly and landed fewer times at the source when hexane rather than ethanol was used, further suggesting that the solvent affected moth flight and orientation behavior. Solvent had no discernible effect on the orientation of *L. botrana* to a piezo-sprayer (El-Sayed et al. 1999b). However, these latter results may not be applicable to all moth and insect species. Furthermore, the vapor pressure of the solvent and/or pheromone used may affect the plume dynamics of the plume released by the sprayer, which may have a resultant effect on moth orientation behavior.

This study demonstrates that the physical setup of the piezo-electric sprayer in a wind tunnel has significant and unpredictable effects on both the amplitude and periodicity of the odor-plume signal produced. Care must be taken when deciding what release rate to use in relation to the configuration of the piezo-sprayer because this is likely to affect the behavioral responses evoked. Furthermore, differences in the plume structure produced by the piezo-sprayer affect the in-flight orientation behavior of male moths, which could affect how strongly they respond to pheromone. These conjectures require confirmation with dedicated experiments. It is also clear that in the future, care must be taken when designing, describing, and interpreting experiments that utilize the piezo-electric sprayer technology.

Acknowledgments We thank Kris Justus and Amy Sage for help with the experimental setup. T. Berhane supervised colony maintenance. This work was supported by Paramount Farms, Inc. and the California Pistachio Commission. We thank Brad Higbee of Paramount Farms for his interest in this project and his suggestions. We are especially grateful to Ashraf El-Sayed for providing much useful advice on setting up piezo-sprayer systems.

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pheromone-mediated mating of parasitoids and even fewer cases where the sex pheromones have actually been identified (Eller et al. 1984, Kainoh et al. 1991; Swedenborg and Jones 1992; Swedenborg et al. 1993; Syvertsen et al. 1995).

Previous work on the pheromone-mediated mating of the aphid parasitoid, *Aphidius nigripes* (Ashmead) (Hymenoptera: Braconidae), reported a diel periodicity in mating. This was modulated by two female pheromones, one that stimulated male upwind flight from a distance, and a second, short-distance one, present in the cuticle of the female (McNeil and Brodeur 1995; Marchand and McNeil 2000). The short-distance component stimulates male courtship behavior when the male is in close proximity to the female but does not elicit upwind flight. Furthermore, there was a significant and permanent reduction in pheromone production after mating (McNeil and Brodeur 1995).

To determine whether the patterns observed hold true for other species in the genus, we undertook a study of the pheromone-mediated mating behavior of *A. ervi* Haliday. *A. ervi* is a solitary endoparasitoid that was introduced in the 1960s into North America from Europe as a biocontrol agent for the pea aphid [*Acrythosiphon pisum* (Harris)] (González et al. 1978), where it is now also found attacking the alfalfa aphid, *Macrosiphum creelii* in certain regions (Michaud and Mackauer 1994). More recently, it has been introduced as a biological control agent into South America and Australia (Schwörer and Völkl 2001). Some research has been done on the foraging behavior of *A. ervi* females (Du et al. 1998; Schwörer et al. 1999; Guerrieri et al. 2002), and on the acceptance of various larval stages as hosts (Sequeira and Mackauer 1994). However, few studies have been conducted on the reproductive biology of this species. Mackauer (1969) compared *A. ervi*'s mating behavior to two other parasitoid species that attack the pea aphid, whereas Battaglia et al. (2002) studied antennation behavior of males during courtship. In this paper, we present: (1) a description of *A. ervi* mating behavior that is mediated by female sex pheromones, and (2) the results from laboratory and field experiments, performed to test the hypotheses that like *A. nigripes*, age and mating status affect both female production of, and male receptivity to, sex pheromones.

Methods and Materials

Insects *Acrythosiphon pisum* and *A. ervi* colonies were established by using material provided by Agriculture Canada. Parasitoids used in these experiments came from mummies collected from aphid-infested broad bean plants, *Vicia faba* L., reared at room temperature (~24°C), under natural light conditions (>15 h light per day).

Newly formed mummies were collected, isolated in individual gelatin capsules, and observed daily for emergence. Each day, the adults were sexed; males and females were placed in separate plastic holding cages with a 20% sucrose solution, and insects were held until needed. In all cases, unless specifically stated, naive virgins of known age were used in all assays.

McNeil and Brodeur (1995) found little evidence of either male or female age affecting the *A. nigripes* male response to extracts of the short-distance pheromone. The absence of age effects was somewhat surprising given that there are significant age-related changes in both the production of, and response to, sex pheromones in Lepidoptera (see McNeil 1991 and references therein). However, the experimental design of the field trials in McNeil and Brodeur (1995) did not allow for a direct assessment of female age effects on the long-distance pheromone. Therefore, in the current study, we included age as a factor in several

of the experiments to test the hypothesis that age would affect both female attractiveness and male responsiveness to pheromones in parasitoids, as reported for moths. We also varied the time of day at which certain assays were conducted to test the hypothesis that mating success, at least in part, was associated with a diel periodicity of both female production of, and male response to, the short-distance pheromone.

Mating Behavior

A newly emerged virgin (<24 h) male and female were paired to observe the behaviors displayed during courtship and copulation. The duration of the pursuit phase, antennation during courtship, and the time spent in copula were measured. To develop an ethogram, 217 naive couples were observed and terminated after 6 min, if mating did not occur, as the probability of copulation was low after 3 min (Mackauer 1969). Observations were made at room temperature between 0700 hours and 1000 hours, as *A. ervi* generally mates in the morning (Mackauer 1969) and, unless specifically stated, all experiments were carried out during this same time period.

In addition the following mating experiments were conducted, to determine:

1. if age affected mating success, by comparing the incidence of mating and time to mate in pairs consisting of a newly emerged virgin male or female with either a newly emerged or 8-day-old member of the opposite sex.
2. the effect of mating status on subsequent mating, by pairing a 1-day-old virgin male or female with a 1-day-old individual of the opposite sex that was either a virgin or that had previously mated on the day of emergence. In the case of females, an additional experiment was carried out, where the incidence of mating of 1-day-old virgins was compared with that of similarly aged females mated <2 h before being paired with 1-day-old virgin males.
3. if there is a diel periodicity in mating activity by comparing mating in pairs of virgin males and females set up at 0800 hours and 1800 hours on the day of emergence.

Twenty-five couples were observed in each experiment and the assays carried out over several days to account for any daily variability due to atmospheric conditions (Marchand and McNeil 2000).

Bioassays with Cuticular Extracts

To establish the optimal extraction time, a preliminary experiment was carried out by soaking newly emerged females in 300 µl of methylene chloride for periods between 180 and 720 s. The 540 s extract gave the highest level of male response (wing fanning behavior) and shortest reaction time following the introduction of the source, so this extraction time was used for all subsequent experiments. Unless specifically stated, all extracts were prepared soon after the lights-on period. Each extract was prepared by using three virgin females, as McNeil and Brodeur (1995) showed that there could be considerable variability in male response to extracts of individual *A. nigripes* females.

Each assay was carried out by using 3 µl [0.01 female equivalents (FE)] placed on a 0.38-cm^2 filter paper disk. All experiments, testing 10 individual 24- to 48-h-old naive virgins, were run between 0700 hours and 1000 hours and replicated three times. The extracts were tested on consecutive days, thereby minimizing any possible daily or atmospheric pressure effects that have been reported in other studies (Lanier and Burns 1978;

Steinberg et al. 1992; Roitberg et al. 1993; Marchand and McNeil 2000). The solvent was allowed to evaporate, and the disk was then introduced into an 8-dr glass shell vial containing a 24- to 48-h-old virgin male parasitoid. Two variables were measured, the proportion of males that responded and the time to respond to the stimulus. Males were considered responsive if they exhibited rapid wing-fanning, the first step in the courtship behavior (Decker et al. 1993; McNeil and Brodeur 1995), within 60 s of exposure to the stimulus.

The following experiments were carried out to determine:

1. the origin of the cuticular pheromone, by comparing the response of 1-day-old virgin males to extracts from the abdomen and the remainder of the body of 1-day-old virgin females.
2. the degree of inter-female variability, by comparing responses to extracts of 10 different <1-day-old virgin females.
3. if there is a diel periodicity in both female production of, and male responsiveness to, the pheromone. The responses of 1-day-old virgin males to female extracts from 1-day-old virgin females, prepared at 0700 hours and 1800 hours, were tested at both 0700 hours and 1800 hours.
4. if female age affected the concentration of pheromone present, by comparing the response of 1-day-old males to extracts from 0-, 2-, 4-, 6-, 8-, 10- and 12-day-old virgin females.
5. if the responsiveness of males was age dependent, by comparing the response of 0-, 2-, 4-, 6-, 8-, and 10-day-old virgin males to a standard pheromone extract from 1-day-old virgin females.
6. to what extent mating affected both attractiveness of, and receptivity to, the cuticular pheromone. To determine attractiveness, the response of 1-day-old virgin males to extracts from 24- to 48-h-old virgin and mated (24 h earlier) females was compared. For receptivity, the responsiveness of 24- to 48-h-old virgin and mated (24 h before) males to an extract from 1-day-old virgin females was compared.

Field Trapping

In all experiments, sticky Delta traps were installed at 10 m intervals, 30 cm above the crop canopy, in an alfalfa plot at Agriculture Canada in London, Ontario. A plastic cylindrical cage (8 cm long and 4 cm diameter), with both ends covered with an open mesh cloth to allow air circulation, was suspended from the roof of each trap. The cages contained a female, with a 20% sugar water solution as a food source or, in the case of controls, just sugar water. All trials were carried out over a 24 h period, starting at 1300 hours, and the number of males captured in each trap was recorded. All parasitoids caught in the sticky traps were collected, sexed, and identified by using the key of the hymenopterous parasites of the pea aphid found in eastern North America (Mackauer and Finlayson 1967). A subsample of those identified as *A. ervi* was sent to Agriculture Canada for confirmation.

The following experiments were carried out, to determine:

1. the level of inter-female variability. Twelve newly emerged females and three controls were randomly distributed along a trap line, and the number of males caught by each female was determined over a 3-day period. Each day, the number of males captured was counted and they were removed, and the position of each trap was re-randomized, to account for any possible effects of trap location.

2. the effect of female age on pheromone production by comparing the attractiveness of newly emerged, 5- and 10-day-old virgin females. Three females in each category and three controls were tested each day. The experiment was replicated four times.
3. if mating affected pheromone production. The attractiveness of 2-day-old females mated on day 1 was compared with that of 2-day-old virgins. Six individuals of each mating category and three controls were tested each day. The experiment was replicated four times.

Statistical Analyses

In the laboratory assays, the proportion of males responding was analyzed by using a chi-square test (α=0.05). For male reaction time as a function of either male or female age, a one-way ANOVA was used, whereas in all other assays, a *t* test was used. For the field trials, the number of males captured in traps containing live females was analyzed by one-way ANOVA.

Results

Mating Behavior

The sequence of events leading to copulation is shown in Fig. 1. In the absence of females, males move actively around the arena, waving their antennae in various directions with their wings held over their abdomen. However, when a female is detected, the male increases walking speed and starts wing fanning. Initially, there are distinct wing fanning bouts, but this behavior becomes almost continuous by the time he contacts the female. The male mounts the female from the back or the side and aligns along her body axis. He advances and places his antennae between those of the female, rapidly moving his own from side to side and making repeated contact with her antennae. This antennal stroking is a prerequisite to successful copulation and probably involves a male contact pheromone present on his antennae that influences female mate choice (see Battaglia et al. 2002). If the female is receptive, she stops moving, raises her abdomen, and positions her antennae laterally, where they remain immobile for the duration of mating. Once the female takes the acceptance position, the male moves backwards and curves his abdomen downwards. Once the genitalia are locked, the female remains completely immobile. Initially, the male continues to slowly antennate the female's antennae/head, but in the final seconds of mating he stops and places his antennae, immobile, above his head. Mating terminates when the female starts walking forward, at which time the male dismounts. Unreceptive females often fly or walk away upon first contact. However, if the male succeeds in mounting an unreceptive female, she continues to move her antennae, flit her wings, lower her head, curve her abdomen forward between her legs, and push the male with her hind legs. About 70% of males tested were successful in mounting the female (Fig. 1), and about 80% of those subsequently succeeded in mating (Fig. 2). If a male was unsuccessful on the first attempt after he mounted the female, he often persisted with antennal stroking, which may occur before or after the first attempt to engage her genitalia. However, the probability of success on subsequent attempts decreases considerably (Fig. 2). Unsuccessful males dismount and move away.

There is a significant effect of both male and female age on male responsiveness (number of males wing fanning, time to wing fanning, number of males attempting to mate)

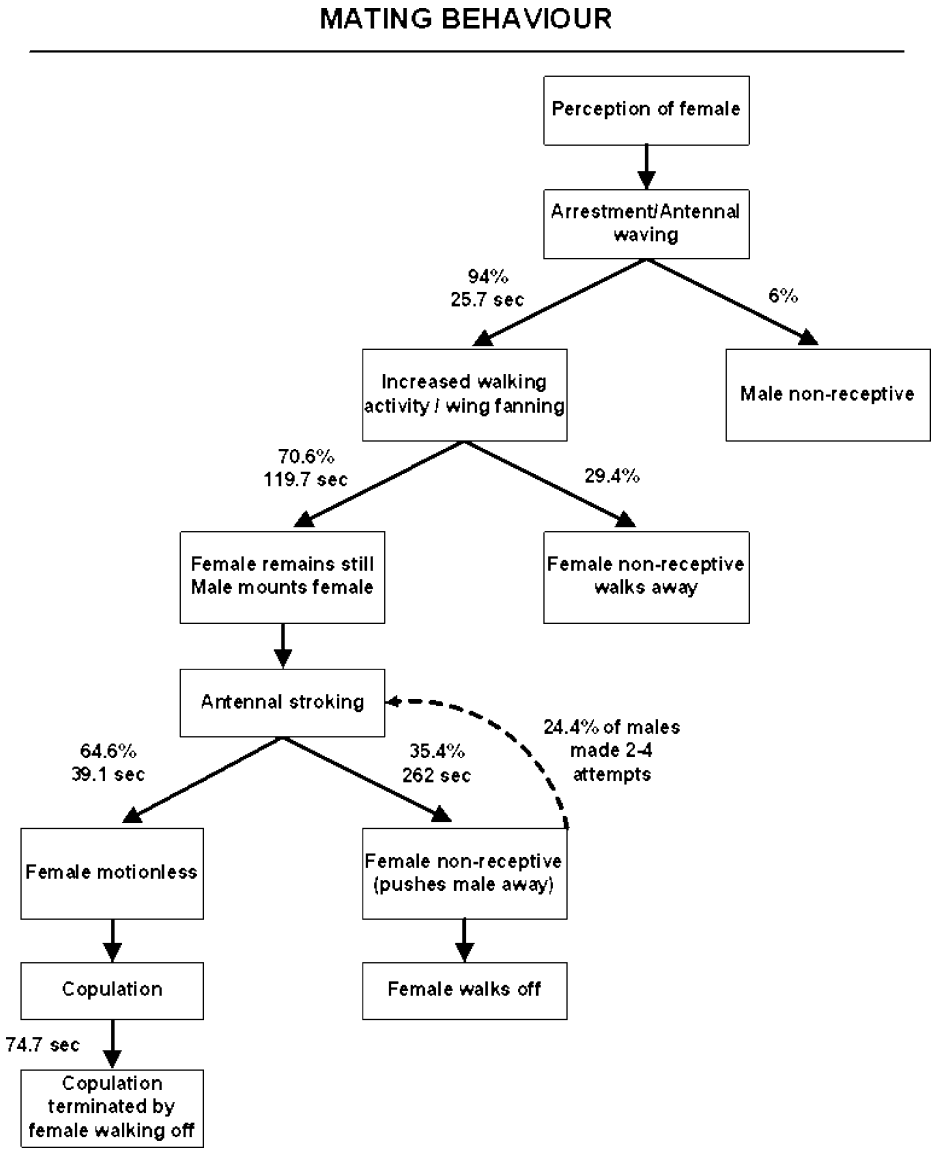


Fig. 1 A flow chart of mating behavior of male *Aphidius ervi* (*N*=217). Percentages indicate the proportion of virgin individuals, <24-h-old, displaying each behavior and the mean duration (in seconds)

and mating success of *A. ervi* (Table 1). There was also a significant effect of time of day on some aspects of male responsiveness and mating success (time to wing fanning, number of males attempting to mate, and number of males succeeding in mating; Table 1). The highest response level and shortest time to respond, as well as incidence of successful mating, were observed with pairs made up of newly emerged adults. Responsiveness and mating success declined, and the time to respond increased, when older males or females were tested, as well as when the bioassays were carried out at the end of the afternoon rather than soon

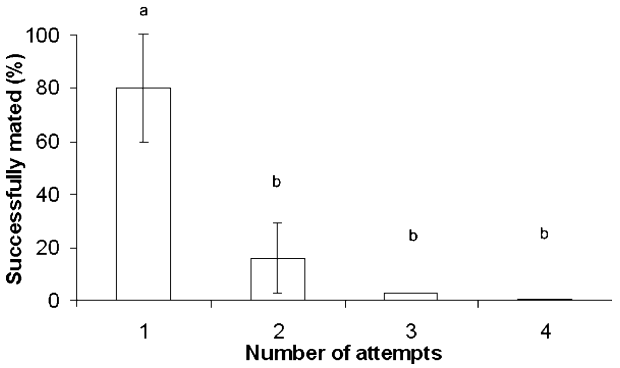


Fig. 2 The incidence of mating success of *Aphidius ervi* males (Mean±SEM), as a function of the number of attempts to copulate with the same virgin female during a courtship bout (N=217). *Bars with different letters* indicated significance at $P<0.05$

Table 1 The effect of female and male age and mating status, as well as time of day assays were conducted, on the number of *A. ervi* males (mean±SE, N=25) that responded to the presence of a female, attempted and succeeded to mate, as well as the time (mean±SE, in seconds) it took them to do so

	Number wing fanning	Time to wing fan	Number attempting to mate	Time to mating attempt	Number succeeding	Duration of copulation
1-day-old virgin female	24	19.7±5.5	17	132.1±36.4	12	73.2±8.3
8-day-old virgin female	18*	47.7±14.3*	5*	90.4±39.7	0*	
1-day-old virgin male	25	20.0±6.7	19	104.4±23.6	11	66.3±5.8
8-day-old virgin male	11*	106.6±45.1*	4*	18.7±5.9*	1*	41.4
1-day-old virgin male	23	23.6±5.6	19	107.5±21.1	14	69.7±6.3
1-day-old mated male	20	62.4±25.1*	13	161.6±51.2*	10	75.7±10.1
1-day-old virgin female	25	22.7±8.4	19	133.1±24.6	13	71.2±5.9
1-day-old female mated 24 h prior	16*	45.6±23.0*	7*	117.2±63.4	4*	99.8±20.2
1-day-old virgin female	24	26.7±10.4	21	113.2±25.0	13	74.4±6.5
1-day-old female mated 2 h prior	17*	19.6±4.2	8*	211.0±83.1*	0*	
1-day-old virgins, tested in a.m.	21	26.8±7.8	17	126.8±23.4	13	73.2±9.5
1-day-old virgins, tested in p.m.	16	16.7±3.2*	9*	160.4±45.5	4*	58.4±13.4

*Significant at $P<0.05$

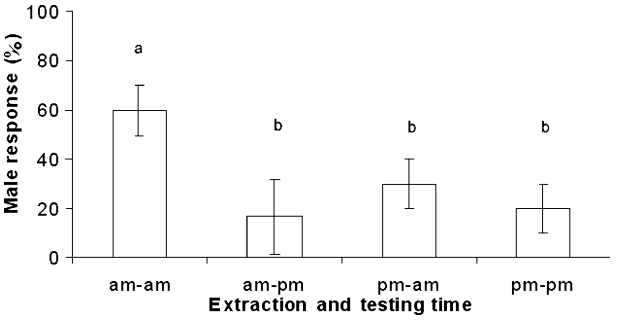


Fig. 3 The proportion of *Aphidius ervi* males (N=30) responding at 7 A.M. and 6 P.M. to 3 μl (0.01 FE) of methylene chloride extracts from 0 to 24-h-old virgin females at the same hours (Mean ±SEM). On the χ-axis, the first value for each trial indicates the time the female extract was made and the second is the time that males were tested. *Bars with different letters* indicate significance at P<0.05

after lights-on (Table 1). The duration of mating varied in the different assays, but no consistent pattern was evident.

Mating, even 2 h before, significantly reduces female attractiveness, as assessed by several indices of male response (Table 1). When males attempted to mate, females generally moved away, exhibited wing flitting and curving of the abdomen forward between her legs. Thus, there was a low incidence of re-mating (Table 1). In no-choice situations, the mating success of previously mated males did not differ significantly from that of similarly aged virgins. It did, however, take significantly longer for them to react to the stimulus, but the mating duration was not significantly different (Table 1).

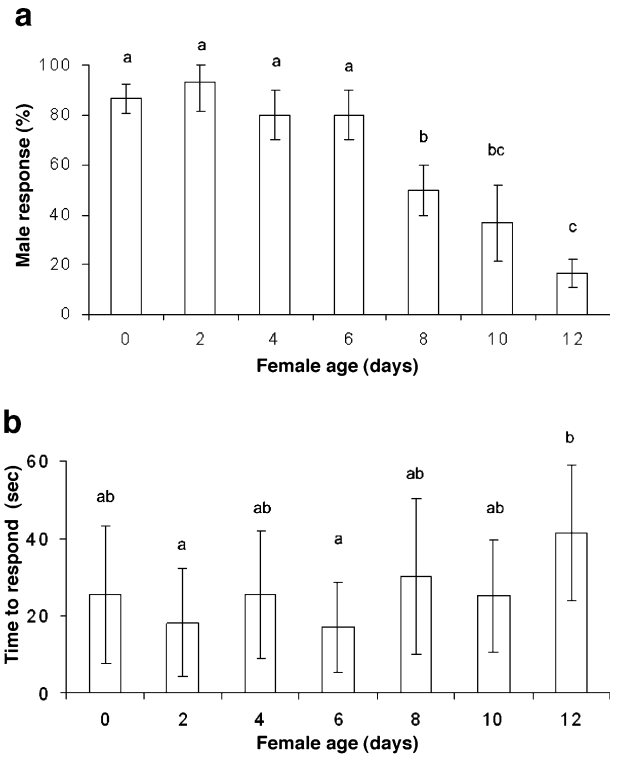


Fig. 4 The effect of female age on **a** the proportion (N=30) of *Aphidius ervi* males responding and **b** the reaction time of responding males to 3-μl (0.01 FE) methylene chloride extracts from virgin females (Mean± SEM). *Bars with different letters* indicate significance at P<0.05

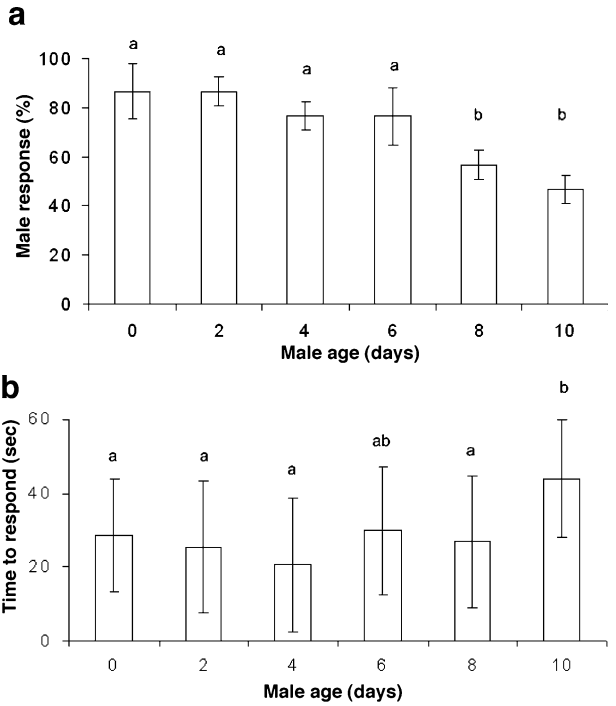


Fig. 5 The effect of male age on **a** the proportion (N=30) of *Aphidius ervi* males responding and **b** the reaction time of responding males to 3-µl (0.01 FE) methylene chloride extracts from 0- to 24-h-old virgin females (Mean±SEM). *Bars with different letters* indicate significance at $P<0.05$

Bioassays with Cuticular Extracts

The extracts of virgin females elicited a strong wing fanning response from males, whereas the whole body extract of a male did not (90 vs 0%). The source of pheromone appears to be in the abdomen, as abdominal extracts elicited higher responses (mean±SE) than those of the head/thorax (66.7±5.8 vs 6.7±11.6%; χ^2=23.254; df=1; $P<0.001$). However, when males responded, the mean reaction time was similar regardless of the origin of the extract (t=2.307; df=20; P=0.144; data not shown). The proportion of males responding to individual female extracts varied, but these differences were not significant (χ^2=12.329; df=9; P=0.195; data not shown), and mean reaction time was similar for all females (F=1.379; df=9,101; P=0.207; data not shown).

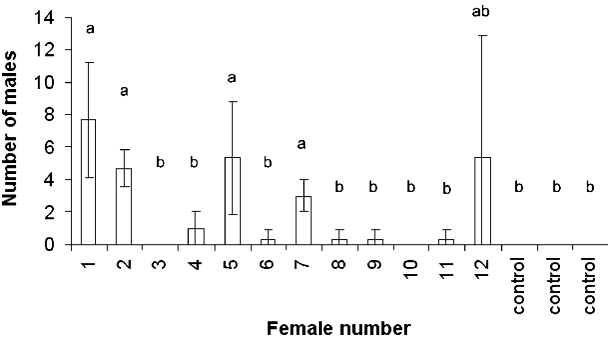


Fig. 6 Evidence of individual variability in the attractiveness of newly emerged virgin *Aphidius ervi* females under field conditions in London, Ontario (Mean±SEM). *Bars with different letters* indicate significance at $P<0.05$

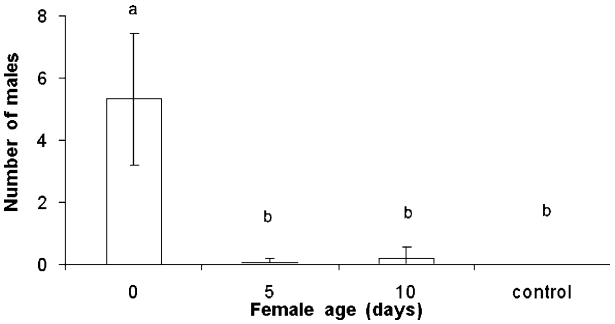


Fig. 7 The effect of age on the attractiveness of virgin *Aphidius ervi* females under field conditions in London, Ontario (Mean± SEM). *Bars with different letters* indicate significance at $P<0.05$

The percentage of males that responded changed depending on the time of day when the female extract was prepared and the time when the assays were carried out ($\chi^2=16.175$; $df=3$; $P=0.001$; Fig. 3). These results suggest a diel periodicity in both female production of the short-distance sex pheromone and the male's receptivity. However, for those males that did respond, their response time did not differ significantly, regardless of the time the extracts were taken or the time the assays were carried out ($F=0.568$; $df=3, 34$; $P=0.640$).

The responsiveness of 24 h-old males to extracts of differently aged virgin females changed with age ($\chi^2=65.455$; $df=6$; $P<0.001$; Fig. 4a), with a noticeable decline occurring with females >6-day-old. This is associated with an increase in time to wing fan, with females >8-day-old ($F=2.533$; $df=6, 114$; $P=0.024$; Fig. 4b). Similarly, male age affected responsiveness to a standard virgin extract, both with respect to the percentage that responded ($\chi^2=19.945$; $df=5$; $P=0.001$; Fig. 5a) as well as the time to respond ($F=3.487$; $df=5, 123P=0.006$; Fig. 5b), and this was due to males >6-day-old.

Extracts from mated females were less attractive than those of similarly aged virgin females (6.6±5.8 vs 86.7±5.8%; $\chi^2=38.571$; $df=1$; $P<0.001$), but as seen in the mating trials, the mean reaction time of those males that responded was unaffected (26.7±22.1 vs 25.6±17.8 s; $t=0.008$; $df=26$; $P=0.929$). In contrast, previous male mating history did not affect the level of response (73.3±5.8 vs 86.7±11.6%; $\chi^2=1.667$; $df=1$; $P=0.197$) or the time to respond (23.8±17.0 s vs 28.6±15.3 s; $t=0.123$; $df=46$; $P=0.727$) to a standard female extract.

Field Trapping

The mean number of males captured per day (ranging from 0 to 8) indicates that there is considerable inter-female variability in the long distance pheromone produced by newly emerged virgins ($F=3.500$; $df=14, 30$; $P=0.002$; Fig. 6). Furthermore, there was a decline

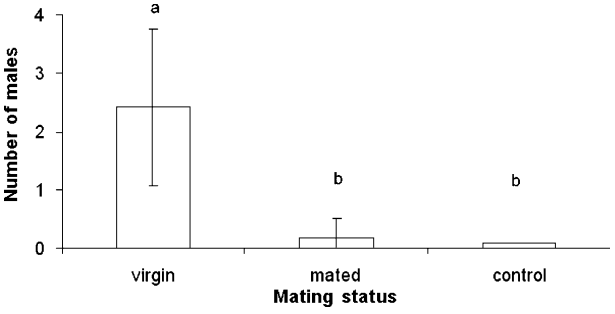


Fig. 8 The effect of mating on the attractiveness of *Aphidius ervi* females under field conditions in London, Ontario (Mean±SEM). *Bars with different letters* indicate significance at $P<0.05$

in the attractiveness of virgin females with age (F=10.014; df=3, 56; P<0.001; Fig. 7). Mating also reduced female attractiveness, with mated females capturing fewer males than virgins of the same age (F=6.092; df=2, 54; P=0.004; Fig. 8).

Discussion

The mating behavior of *A. ervi* is similar to that of *A. nigripes*, with female pheromones modulating attraction and courtship behavior (McNeil and Brodeur 1995; Marchand and McNeil 2000), and with both short- and long-distance pheromones. The short-distance pheromone affecting courtship (wing fanning and attempted copulation) is located in the female abdomen and, as suggested for *A. nigripes*, the low levels of response observed to head/thorax extracts may result from female grooming behavior (McNeil and Brodeur 1995). In both species, the production of, and response to, the short-distance pheromone shows diel periodicity, being maximal in the morning. In *A. ervi*, this periodicity was also evident in the mating assays, with the number of attempted and successful matings being significantly higher in the morning than in the afternoon. Similarly, there was evidence of diel periodicity associated with the long-distance pheromone in *A. nigripes*, as the majority of males captured in traps baited with virgin females occurred between 0600 hours and 1000 hours (McNeil and Brodeur 1995). Mating early in the morning would be quite advantageous for small insects like *A. ervi*, as the cool temperatures and high relative humidity reduce the risk of desiccation. Furthermore, the generally calmer wind conditions at this time of day would be good for foraging males, as flight is inhibited by winds >1 m/s (Marchand and McNeil 2000; McClure and McNeil unpublished data).

A number of parameters affect female production/emission of sex pheromones in *A. ervi*. For example, field trials provide evidence of individual variability in the production of the long-distance pheromone although, while variation was observed, there were no significant differences in the laboratory bioassays that tested the short-range pheromone. A similar pattern of individual variation between females was noted for *A. nigripes*, in field (McNeil and Brodeur 1995), as well as in wind tunnel experiments where female body size (related to the size of the host) played a significant role (Cloutier et al. 2000). There was a significant effect of age on long-distance attraction by *A. ervi* females, presumably due to a decline in pheromone production as reported for the age-related decline in pheromone production in virgin *Helicoverpa zea* females (Teal et al. 1990). There was also a decline in male responsiveness to short-range pheromone as a function of female age, but only to extracts from individuals >6 day-old, similar to the non-significant trend observed for *A. nigripes* (McNeil and Brodeur 1995). The results of this study support the hypothesis that female age affects pheromone production in parasitic Hymenoptera, especially the long-distance one that stimulates male upwind flight.

It would also appear that females of *Aphidius* species are generally monandrous and that repeated matings are rare (results of this study, McNeil and Brodeur 1995). Thus, a post-mating decline in pheromone production (pheromonostatis) is not surprising, and has previously been suggested for other parasitoids (Van den Assem 1986). This phenomenon has generally been studied in Lepidoptera (e.g., Raina et al. 1994; Ramaswamy et al. 1996; Marcotte et al. 2003, 2005), and Diptera (e.g., Ottiger et al. 2000), and the length of the female refractory period may be related to a number of factors, including the quality of the male ejaculate (see Kingan et al. 1993, 1995; Fan et al. 1999a,b; Delisle and Bouchard 1995; Delisle and Hardy 1997; Marcotte et al. 2005). Few *A. ervi* females re-mated, and as

it was only observed in those that had mated 24 h earlier, re-mating was possibly the result of an unsuccessful first mating or due to a low quality mate.

There appear to be fewer, or less-pronounced, changes in the responsiveness of *A. ervi* males to the female short-distance pheromone. There is a decline in responsiveness by males >6 day, which could be the result of reduced sensitivity of peripheral pheromone receptors and/or the integration at the level of the central nervous system (see Hansson 1999). Furthermore, the mating success of 8-day-old males that do respond to the sex pheromone is significantly lower than for 1-day-old individuals, possibly associated with the production of male infochemicals involved in female mate choice (Battaglia et al. 2002). However, while the responsiveness of young, previously mated *A. ervi* males is significantly slower than similarly aged virgins, previous mating history does not affect acceptance by females in a no-choice situation. Whether this is true in a competitive situation, or when older virgin males are involved, remains to be determined. Furthermore, even if females did not discriminate the longer response time of previously mated males, this could result in them being out-competed by virgin conspecifics.

Additional research is required to better understand the causes of the observed changes in the emission of, and response to, sex pheromones in *A. ervi*, as well as to determine to what extent they impact reproductive success. Furthermore, we need to know how factors such as temperature, wind speed, and humidity affect the pheromone-mediated communication system. The information generated would not only add to our basic understanding of insect reproductive biology, but could help determine the optimal conditions for parasitoid release and their effective deployment in pest management programs. Furthermore, the chemical composition of these pheromones must be elucidated if one wishes to test their potential in "push–pull" management strategies (Pyke et al. 1987; Miller and Cowles 1990; Khan et al. 2000; Nalyanya et al. 2000).

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Keywords *Porotermes adamsoni* · *Stolotermes victoriensis* · Foraging behavior · Chemical communication · Sternal gland · Phylogeny · (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol

Introduction

Pheromones form the mechanistic basis of the organization of foraging in termites, which is the most significant factor affecting their social evolution as emphasized by Traniello and Leuthold (2000) in their review of behavior and ecology of foraging in termites. Termite trail-following pheromones are relatively well known for the Rhinotermitidae and Termitidae of the 'separate life type' (Abe 1987), i.e., for species in which workers forage outside the nest or move from one nesting site to another. In these termites, trail pheromones elicit recruitment and orientation (Traniello 1982; Traniello and Leuthold 2000). Their major component is either an unsaturated straight chain C12 alcohol or a diterpene hydrocarbon (Pasteels and Bordereau 1998; Wobst et al. 1999; Peppuy et al. 2001a, b; Robert et al. 2004; Sillam-Dussès et al. 2005).

In contrast, little is known about those termites that feed on wood in which they also nest ('one-piece life type'; Abe 1987) and, a priori, may have less need for a trail pheromone, but some species of the one-piece nesting termite *Prorhinotermes* may leave their nests (Roisin et al. 2006). The first trail-following pheromone of termites was nonetheless demonstrated in a 'one-piece life type' dampwood termite, *Zootermopsis nevadensis*, (Lüscher and Müller 1960; Stuart 1961), and originally identified incorrectly as hexanoic acid by Hummel and Karlson (1968) and Karlson et al. (1968). The true pheromone structure was recently identified as 4,6-dimethyldodecanal (Bordereau et al. 2006), which is chemically different from trail pheromones of more advanced termites. According to Stuart (1963, 1967, 1969), trail pheromones of the 'one piece life type' termites possibly should be considered more as alarm signals eliciting excitation and recruitment for the defense of the colony. The sternal gland secretion also may have other functions, such as a fungistatic activity (Rosengaus et al. 2004).

In the framework of an evolutionary study on the strategies of chemical communication in termite foraging, the behavioral and chemical properties of sternal gland secretions were investigated in basal termites. The sternal gland, a small ectodermal abdominal gland, is the only glandular source for trail pheromones (Noirot 1969).

We report here the results obtained on *Mastotermes darwiniensis*, which represents the most basal lineage among extant termites, although it exhibits a mixture of primitive and advanced characters, especially with the presence of a true worker caste. *Mastotermes darwiniensis*, endemic to northern Australia, is a unique living representative of a family of termites distributed worldwide in the past. Its phylogenetic relationships with the subsocial cockroach *Cryptocercus* often have been emphasized, but they remained controversial (Thorne 1990, 1991; Nalepa 1991; Grandcolas 1996; Grandcolas and Deleporte 1996; Nalepa and Bandi 2000; Nalepa and Lenz 2000; Lo et al. 2000, 2003; Eggleton 2001) until Inward et al. (2007) showed from a molecular phylogenetic study that *Cryptocercus* is indeed the sister group of termites. *Mastotermes darwiniensis* is the only extant termite whose workers possess three sternal glands located on sternites 3–5. All other termites possess only one sternal gland located on the fourth or fifth sternite. The architecture of its subterranean nest is not well known, but colonies typically feed on multiple trees over extensive areas, requiring extensive subterranean galleries (Miller 1993; Goodisman and Crozier 2002). This termite is, therefore, of an intermediate ecological life type, suggesting

that it might use a trail pheromone. However, a trail pheromone has not been demonstrated previously in this basal termite. Colonies of *M. darwiniensis* in natural woodlands are relatively moderate in size, but huge colonies composed of several million individuals can be found in disturbed habitats, such as urban areas, plantations, and the like, where they may cause severe damage (Hill 1942; French 1986).

We also extended our study to two other basal termites with uncertain phylogeny, *Porotermes adamsoni* (Termopsidae, Porotermitinae) and *Stolotermes victoriensis* (Termopsidae Stolotermitinae; Emerson 1942, 1947; Kambhampati and Eggleton 2000). There are only three species of Porotermitinae in the world; their present geographical distribution (Chile, South Africa, Australia) suggests a Gondwanian origin from the Triassic period. *Porotermes adamsoni*, a unique representative of Porotermitinae in Australia, is considered a relict species. This dampwood termite from south-eastern Australia, generally considered as a 'one-piece ecological life type' termite, although, according to Nkunika (1988), its pseudergates are able to build subterranean galleries and can move from one piece of wood to another. Its colonies are composed of a few thousand to tens of thousands of individuals (Mensa-Bonsu 1976; Lenz 1994). *Stolotermes victoriensis* shares much of its range with *P. adamsoni* in mountainous regions of south-eastern Australia. Its phylogenetic position remains controversial. Although generally considered a basal termite, the configuration of the genitalia would indicate a closer relation of Stolotermitinae with Kalotermitidae, Rhinotermitidae, and Termitidae rather than with Termopsinae and Hodotermitidae (Klass et al. 2000). *Stolotermes victoriensis* is also a 'one-piece life type' termite with pseudergates. Its colonies are composed of a few hundred small individuals.

Thus, the principal objectives of this research were threefold: (a) to investigate whether trail pheromones were present and secreted from the sternal glands in the basal termites *M. darwiniensis*, *P. adamsoni*, and *S. victoriensis*; (b) to identify, synthesize and confirm by bioassay any trail pheromones arising from such studies; and (c) to correlate these results with termite phylogeny.

Materials and Methods

Insects

Mastotermes darwiniensis termites were collected in the field in Australia (Darwin in the Northern Territory and Townsville in Queensland), or were reared in the laboratory in Canberra (ACT) and in Dijon (France). The termites used in Canberra were collected in the Northern Territory and reared in metal drums in climate-controlled rooms (30°C, 90% RH). Most termites used in Dijon came from a colony founded in Australia and taken in 1973 to the Federal Institute of Testing of Materials (BAM) in Berlin (Germany; M. Lenz) and maintained in Dijon since 1999 (28°C, 80% RH). Incipient colonies founded in Darwin in 1999 from swarming imagos, then reared in Dijon, were also used. Workers of *M. darwiniensis* are of large size (9–10 mm long). They possess three sternal glands, of about 0.006 mm^3 for the first two, and a maximum of 0.001 mm^3 for the third.

Porotermes adamsoni termites were collected from stumps or logs of *Eucalyptus* in the Brindabella Mountains near Canberra. They were used in Canberra for trail-following bioassays shortly after collection, or later in Dijon where they were maintained in a climate-controlled room (26°C, 60% RH). Pseudergates of this species also are of large size (8 mm long) and their unique sternal gland is well developed (about 0.03 mm^3).

Stolotermes victoriensis is a termite colonizing wet and decayed logs in southeastern Australia. Pseudergates are 6.5 mm long, and the volume of their sternal gland is about 0.001 mm^3. The termites were collected in the Brindabella Mountains near Canberra, and then maintained in Dijon until use.

Pheromone Extracts

The pheromone was extracted by using two procedures. (a) Whole bodies or sternal glands of workers or pseudergates were immersed in different purified solvents (pentane, hexane, dichloromethane, or methanol) for 12 h at 4°C. After removing workers or glands, extracts were stored at −20°C until used for bioassays or chemical analyses. Sternal glands were prepared by dissecting the glands from cold anesthetized individuals under a stereomicroscope with microscissors and forceps. They were extracted in 2 ml of solvent for 500 glands. (b) Cold anesthesized termites were artificially induced to expose their sternal glands under a stereomicroscope by stretching the abdominal segments with a forceps. A Supelco 65 μm-fiber for solid phase microextraction (SPME) was gently rubbed on the sternal glands. Different types of fibers were tested; the polydimethylsiloxane/divinylbenzene type gave consistently best results. Surface chemicals from 30 individual termites were accumulated on the fiber before thermal desorption. Controls were carried out by rubbing other intertergal membranes or tergites with the same type of SPME fiber. The fiber was desorbed in the injection port of a gas chromatograph for 3 min for gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses.

Fractionation of extracts of sternal glands of *M. darwiniensis* and *P. adamsoni* was carried out by preparative GC (Hewlett-Packard HP 6890 GC) connected to a Gerstel preparative fraction collector, using an HP5 capillary column (30 m×0.32 mm ID) (0.25-μm film thickness). The GC effluent was trapped in 120-μl glass U-tube traps cooled in liquid nitrogen. Fractions were collected from a total of 150 individuals, the SPME fiber being desorbed in the GC after rubbing the sternal gland of 30 individuals. The biological activity of fractions was tested in trail-following bioassays.

Cuticular Hydrocarbons

Cuticular hydrocarbons were obtained by extracting 100 workers in 5 ml hexane for 1 h at 4°C, then purifying the extract on silica gel columns (hexane elution).

Chemical Analyses

GC and GC-MS analyses were carried out with a 5973N Mass Selective Detector coupled to a 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with a split-splitless injector and a DB™-Wax column (30 m×0.32 mm ID, 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA) or an Equity 5 column (30 m×0.32 mm ID, 0.25 μm film thickness, Supelco). Columns were heated from 40 to 240°C at 5°C min^{-1}. Helium was used as carrier gas at a constant velocity of 37 cm/s. The temperature of the injector was set to 250°C. The column was interfaced directly to the ion source of the mass spectrometer through a heated transfer line maintained at 250°C. Electron-ionization (EI) mass spectra were obtained at 70 eV with the instrument scanning from *m/z* 29 to 450, and the source maintained at 230°C.

Positive chemical ionization (PCI) mass spectra were generated with ammonia as reagent gas (PCI-NH_3). The instrument was scanned from *m/z* 60 to 300 in a 0.7 s cycle.

Quantification of the Pheromone

Because SPME is not a satisfactory quantitative method of extraction, the quantity of pheromone was estimated from a comparison of GC peaks of the pheromone present in sternal gland extracts and of the standard. Due to the limited biological material available in France, the measurements were made on two extracts of 500 sternal glands in *M. darwiniensis* and 100 sternal glands in *P. adamsoni* and *S. victoriensis*. Therefore, it was not possible to use statistical analyses. These qualitative estimates were then compared to the data from the trail-following bioassays.

Synthesis of (*E*) and (*Z*)-2,6,10-Trimethyl-5,9-undecadien-1-ol

Racemic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol [containing 1.5% (*Z*)-isomer] was prepared by way of the Darzens condensation of geranylacetone (Fluka, Sydney) with ethyl chloroacetate followed by hydrolysis of the intermediate ester, thermal decarboxylation, and $LiAlH_4$ reduction of the resultant aldehyde, as adapted from the method of Kulesza and Gora (1969). (*Z*)-2,6,10-Trimethyl-5,9-undecadien-1-ol [containing 2% (*E*)-isomer] was prepared similarly from nerylacetone (Fluka, Sydney).

Trail-Following Bioassays

Bioassays were carried out either in Canberra with termites freshly collected from the field or in Dijon with laboratory-reared termites. All bioassays were performed in climate-controlled rooms (30°C, 80% RH) under red light. The orientation activity elicited by extracts was assayed with two types of bioassays.

(a) Trail extracts were tested with a 'Y open field' trail-following bioassay on Whatman no.1 filter paper discs (15-cm diameter), with a 120° angle between each branch. On the Y stem (3 cm) and one of the Y branches (7 cm), an artificial trail was drawn with a microliter syringe containing 1 μl of extract per cm of trail. Another extract or solvent as a control was deposited under the same conditions on the base and the other branch of the Y. One termite was placed inside a holding chamber consisting of a small plastic vial (55 mm ID), with the 2-mm wide opening being located at the base of the Y. The distance traveled by the insect on the trail was measured. The activity threshold for an extract was defined as the minimum concentration that elicited termites to travel a mean distance of more than 3 cm, the maximum response being 10 cm. Each termite and each trail were used only once to prevent any effects from behavioral conditioning or trail reinforcement.

(b) Trail extracts were also tested with a 'T maze' bioassay. T-shaped artificial trails were drawn on a Whatman no.1 filter paper and covered with a T tunnel formed in a perspex block laid on the filter paper disc. The T base was 3 cm long, and the branches 6 cm long. The termites were thus guided by the tunnel and had the choice between the left and the right branches.

Exploratory Behavior of a New Environment

The behavior of termites while exploring a new environment was observed by using an 'open field' design: a piece of wood containing a few hundred individuals was deposited at

the center of a sheet of filter paper (40×60 cm) covering the bottom of a plastic container. Observations of termites exploring their surroundings were made under red light.

Statistical Analyses

Results of trail-following bioassays in the open field design were analyzed with a *t* test, whereas those in the T-maze were analyzed with a Kruskall-Wallis or χ^2 test. In this latter case, results were considered significantly different when choices were ≥21/9 for N=30 (significance set at 0.05).

Results

Exploratory Behavior and Trail-following Pheromone

Mastotermes darwiniensis On a clean arena, workers of *M. darwiniensis* explored their environment individually and in tandem and were never seen moving in single file as do termites known for secreting a trail pheromone (Fig. 1a). Moreover, in an open field

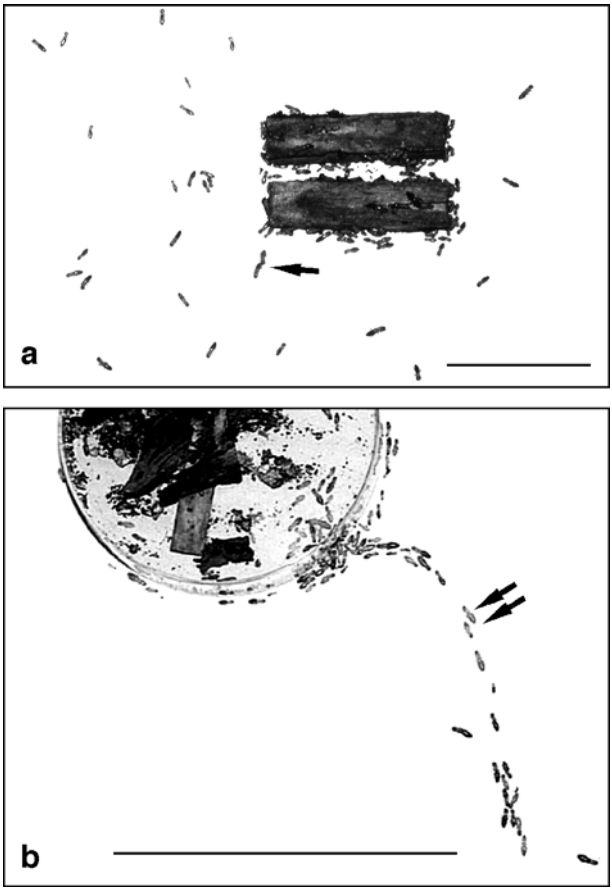


Fig. 1 **a** Exploratory behavior of termites in a new environment. Deposited at the center of a clean container, workers of *M. darwiniensis* scatter over the whole surface and explore their environment individually or in tandem (*arrow*) (scale bar=10 cm). **b** Under the same experimental conditions as for workers of *M. darwiniensis*, pseudergates of *P. adamsoni* explore their environment in single files (*double arrows*), showing the use of a trail pheromone and mass recruitment (scale bar=10 cm)

situation, workers were unable to follow artificial trails made from extracts of whole workers or sternal glands, irrespective of the solvent used for extraction. Even at a concentration of five sternal glands/cm of trail, trail-following did not reach the activity threshold of 3 cm (mean±SD: 1.9±0.5 cm, N=30). These observations raised doubts whether *M. darwiniensis* was secreting a trail-following pheromone. However, T-maze bioassays (Table 1) showed that the workers of *M. darwiniensis* were able to distinguish a trail used by nestmates from a control trail. When given a choice between a control trail and a trail previously followed by at least 50 nestmates, workers preferentially chose the previously used trail, indicating that workers actively or passively mark the substrate when moving.

Workers of *M. darwiniensis* also were able to follow an artificial trail made of hexane extracts of whole workers of at least five individuals/cm of trail in a 'guided' context, indicating a chemical origin of the 'marking' (for 30 tests, 28 choices for the trail made from worker extracts, 2 choices for the control trail made from hexane). Moreover, when given a choice between a trail made with extracts of whole workers without sternal abdominal segments and a trail made from sternal glands 3–5, workers preferentially chose the trail made with sternal gland extracts (26 choices out of 30 tests). In contrast, a trail consisting only of cuticular hydrocarbons of workers was not followed (for 30 tests, 18 choices for the trail made from cuticular hydrocarbons at a concentration of 1 worker equivalent/cm, 19 choices at a concentration of 5 worker equivalent/cm). These results suggest that *M. darwiniensis* secretes a trail-following pheromone from its sternal glands as do other species of termites.

Extracts of sternal gland 4 were about five times more active than those of sternal gland 3 (Table 2). This result was confirmed with a choice test between trails made from extracts of sternal gland 4 and sternal gland 3, at a concentration of 1 gland/cm; workers clearly preferred the trail with the sternal gland 4 extract. Trail-following was not observed for extracts of sternal gland 5, probably due to its very small size.

Porotermes adamsoni In contrast to the individual exploratory behavior of *M. darwiniensis*, pseudergates of *P. adamsoni* explored a new environment in single file with individuals following each other closely (Fig. 1b). This behavior suggested the use of trail pheromone. In contrast also to *M. darwiniensis*, pseudergates of *P. adamsoni* were able to follow artificial trails in an open field situation, secreting a very active trail pheromone from their abdominal sternal gland. The biological activity threshold of trails made from sternal gland was 10^{-2} gland/cm (mean±SD: 7±0.7 cm, N=30). Trail-following was optimal at 10^{-1} and 1 gland/cm (mean: 10 cm, n=30, and reduced at higher concentrations (5 sternal gland/cm; mean±SD: 7.5±0.5 cm, N=30). This bell-shaped dose-curve or concentration-response is characteristic of pheromones.

Table 1 Results of a T-maze trail-following bioassay. Workers of *M. darwiniensis* are able to recognize a trail previously used by at least 50 nestmates. (S: result statistically significant, NS: result statistically non significant, N=30)

Number of Passages	Number of Choices		χ^2 test
	Control	Trail	
25	14	16	NS
50	8	22	S
100	9	21	S
250	6	24	S
1,000	4	26	S

Table 2 Comparison of the trail-following activity of sternal glands 3 and 4 in *M. darwiniensis*

	Trail Concentration	1 Weq/cm	0.5 Weq/cm	0.25 Weq/cm	0.1 Weq/cm
Choices	Sternal gland 3 vs hexane	26 vs 4 (S)	22 vs 8 (S)	17 vs 13 (NS)	17 vs 13 (NS)
	Sternal gland 4 vs hexane	28 vs 2 (S)	–	–	26 vs 4 (S)
	Sternal gland 3 vs Sternal gland 4	7 vs 23 (S)	–	–	–

Extracts of sternal gland 4 (active at 0.1 Weq/cm) were at least five times more active than extracts of sternal gland 3 (active at 0.5 Weq/cm)

S, result statistically significant, *NS*, result statistically non significant, *Weq*, worker equivalent, *N*=30

Stolotermes victoriensis Pseudergates of *S. victoriensis* also followed artificial trails in the open field bioassay. Bioassays showed that the sternal gland was the only glandular source of the trail pheromone. Optimal trail-following was elicited at a concentration of 10^{-1} gland/cm of trail.

Interspecific trail-following bioassays between *M. darwiniensis*, *P. adamsoni*, and *S. victoriensis*

Interspecific trail-following bioassays showed positive responses between *M. darwiniensis*, *P. adamsoni*, and *S. victoriensis*. In a T-maze bioassay, workers of *M. darwiniensis* followed an artificial trail made from extracts of sternal glands of *P. adamsoni* pseudergates (Table 3). Moreover, when given a choice between trails prepared from extracts of *M. darwiniensis* or *P. adamsoni* sternal glands, workers of *M. darwiniensis* preferentially chose the trails of *P. adamsoni* (Table 3).

Pseudergates of *P. adamsoni* always chose the *P. adamsoni* trail when given a choice between *M. darwiniensis* and *P. adamsoni* sternal gland extract trails (Table 3). However, in 'open field' assays, they were able to follow artificial trails made of *M. darwiniensis* sternal gland but to a lesser degree than their own trails (Table 4).

In T-maze bioassays, pseudergates of *S. victoriensis* were not able to distinguish a trail of *Mastotermes* sternal gland extract from their own (Table 3), and in an open field assay, they followed a *Mastotermes* trail better than their own (Table 4).

In sum, these results suggest the presence of a trail-following pheromone component common to *M. darwiniensis*, *P. adamsoni,* and *S. victoriensis*.

Table 3 Interspecific trail-following in a T-maze bioassay

	Number of Individuals Following Each Trail				
Tested Species	Hexane	*P. adamsoni*	*M. darwiniensis*	*S. victoriensis*	χ^2
M darwiniensis	5	25	–	–	S
M. darwiniensis	–	24	6	–	S
P. adamsoni	–	30	0	–	S
S. victoriensis	–	–	17	13	NS

Workers of *M. darwiniensis* were able to follow trails of sternal gland extracts of *P. adamsoni* and that they preferentially chose trails made from extracts of *P. adamsoni* rather than their own trails

Pseudergates of *P. adamsoni* preferred their own trails to trails of *M. darwiniensis*. Pseudergates of *S. victoriensis* followed their own trails and trails of *M. darwiniensis* without preference (concentration of trails: five sternal glands/cm; *N*=30)

S, result statistically significant; *NS*, result statistically non significant

Table 4 Results of interspecific 'open-field' trail-following bioassays

Tested Species	Trail Following in cm (mean ± SD)			
	Hexane	*P. adamsoni*	*M. darwiniensis*	*S. victoriensis*
P. adamsoni	0	8.4±0.5	–	–
	0	–	5.1±0.5	–
S. victoriensis	0	–	10	–
	0	–	–	6.9±1

Pseudergates of *P. adamsoni* and *S. victoriensis* were able to follow trails of sternal glands of *M. darwiniensis* (N=30, Concentration of trails: five sternal glands/cm)

Chemical Nature of the Trail Pheromone

The existence of a compound common to the three species was confirmed by GC-MS after SPME of the surface of the sternal glands (Fig. 2). It was present in relatively high quantities in *P. adamsoni*, and in lesser amounts in *M. darwiniensis* and *S. victoriensis*, where it was detected only by selected ion monitoring to increase sensitivity. Moreover, it partly co-eluted with another compound in *M. darwiniensis*, but its presence was confirmed in two samples of 500 dissected sternal glands.

For *M. darwiniensis* and *P. adamsoni*, the accumulated SPME extracts of the sternal gland surfaces of 150 individuals were separated into three fractions by preparative GC. Fraction F1 from retention times 3 to 29.40 min comprised the most volatile compounds; fraction F2 from RT 29.40 to RT 29.60 min contained only the common compound specific to the sternal gland surface; fraction F3 from RT 29.60 to RT 48 min contained the less volatile compounds, essentially cuticular hydrocarbons from C23 to C28 for *M. darwiniensis*, and from C22 to C30 for *P. adamsoni.* Results in Table 5 show that workers of *M. darwiniensis* followed only the

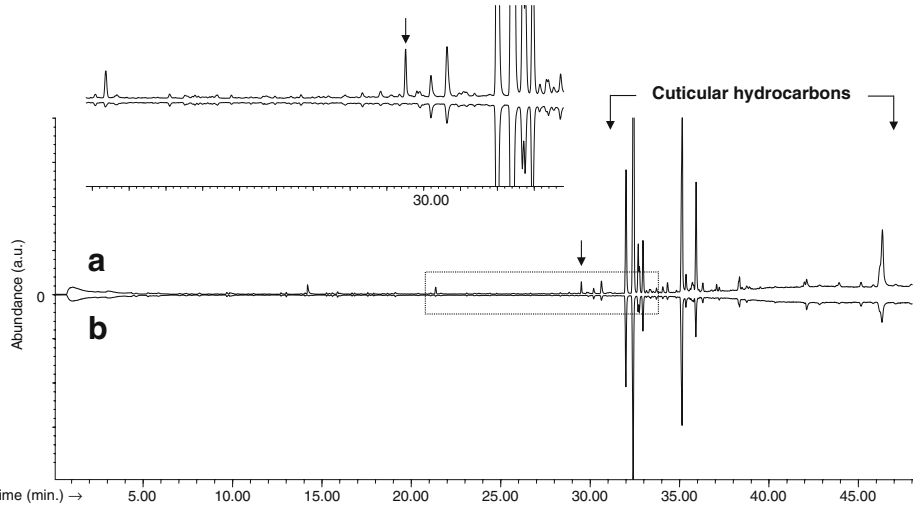


Fig. 2 Comparison of the GC profiles of SPME extracts of (*a*) the surface of sternal gland 5, and (*b*) the extra-glandular abdominal integument of 50 pseudergates of *P. adamsoni*. The majority of peaks are common to both surfaces and correspond to cuticular hydrocarbons from C22 to C30. However, the peak marked by an arrow (RT 29.5 min) is specific to the glandular surface. Detail of the window in the upper part of the figure

Table 5 Trail-following activity of preparative GC fractions of SPME extracts of the sternal gland surface from 50 individuals in *M. darwiniensis* and *P. adamsoni*

	Extracts of *M. darwiniensis* Conc: 0.5×10^{-1} ng/cm (n=30)			Extracts of *P. adamsoni*					
				Conc: 10^{-1} ng/cm (n=30)			Conc: 0.5×10^{-1} ng/cm (n=30)		
Tested species	F1/ hexane	F2/ hexane	F3/ hexane	F1/ hexane	F2/ hexane	F3/ hexane	F1/ hexane	F2/ hexane	F3/ hexane
M. darwiniensis	18/12 (NS)	21/9 (S)	15/15 (NS)	19/11 (NS)	29/1 (S)	17/13 (NS)	–	–	–
				Open field trail-following (cm; n=15)					
P. adamsoni	14/16 (NS)	21/9 (S)	18/12 (NS)	1.3±0.3	5.9±0.9	1.5±0.4	1.8±0.4	10	7.8±1.1

Fraction F1 (0 to 29.40 min); Fraction F2 (29.40 to 29.60 min)=compound specific to the sternal gland surface; Fraction F3 (29.60 to 48 min)=cuticular hydrocarbons; concentration of 10^{-1} ng/cm

Conc, concentration of the trail; *S*, result statistically significant; *NS*, result statistically non significant

trails made of fraction F2 prepared either from *M. darwiniensis* or from *P. adamsoni*. Trail-following was better with trails made from the *P. adamsoni* fraction F2.

In the same manner, pseudergates of *P. adamsoni* followed only the trails made from fraction F2 of the *M. darwiniensis* sternal gland extracts (Table 5). In open field bioassays with trails made of sternal gland extracts of *P. adamsoni*, only the trails made with fraction F2 were active in eliciting trail-following in pseudergates of *P. adamsoni* when tested at the concentration of 10^{-1} ng/cm. They were also the most active when tested at higher concentration (5×10^{-1} ng/cm), eliciting maximal trail-following, but it was noted that at this concentration, fraction F3 containing the cuticular hydrocarbons was also active (Table 5).

The common sternal gland compound of the three species had a retention index (RI) of 2161 on a polar DB-Wax column, and a RI of 1593 on a nonpolar Equity 5 column. Its EI mass spectrum (Fig. 3) suggested a terpenoid structure similar to that of 3,7,11-trimethyl-6,10-dodecadien-1-ol (Bergström et al. 1968). The molecular ion M^+ of *m/z* 210 was confirmed by positive ion chemical ionization (PCI/NH_3), which displayed an MH^+ ion of *m/z* 211. These results suggested that the compound might be the norsesquiterpene homolog of 3,7,11-trimethyl-6,10-dodecadien-1-ol, i.e., 2,6,10-trimethyl-5,9-undecadien-1-ol, which was confirmed by the synthesis of the molecule. The synthetic (*E*)-isomer of 2,6,10-trimethyl-5,9-undecadien-1-ol possessed the same EI mass spectrum and RI (RI 2163 on DB-Wax, and 1598 on Equity 5) as the natural compound. The (*Z*)-isomer was resolved clearly from the (*E*)-isomer (RI 2137 on DB-Wax, and 1572 on HP5). No trace of (*Z*)-2,6,10-trimethyl-5,9-undecadien-1-ol was detectable in extracts of *M. darwiniensis* and *P. adamsoni*.

Biological Activity of Synthetic (*E*) and (*Z*) Isomers of 2,6,10-Trimethyl-5,9-undecadien-1-ol

Mastotermes darwiniensis (*E*)-2,6,10-Trimethyl-5,9-undecadien-1-ol elicited significant trail-following at a concentration of 1 ng/cm of trail, whereas trail-following was reduced beyond a concentration of 100 ng/cm (Table 6). Surprisingly, similar results were obtained

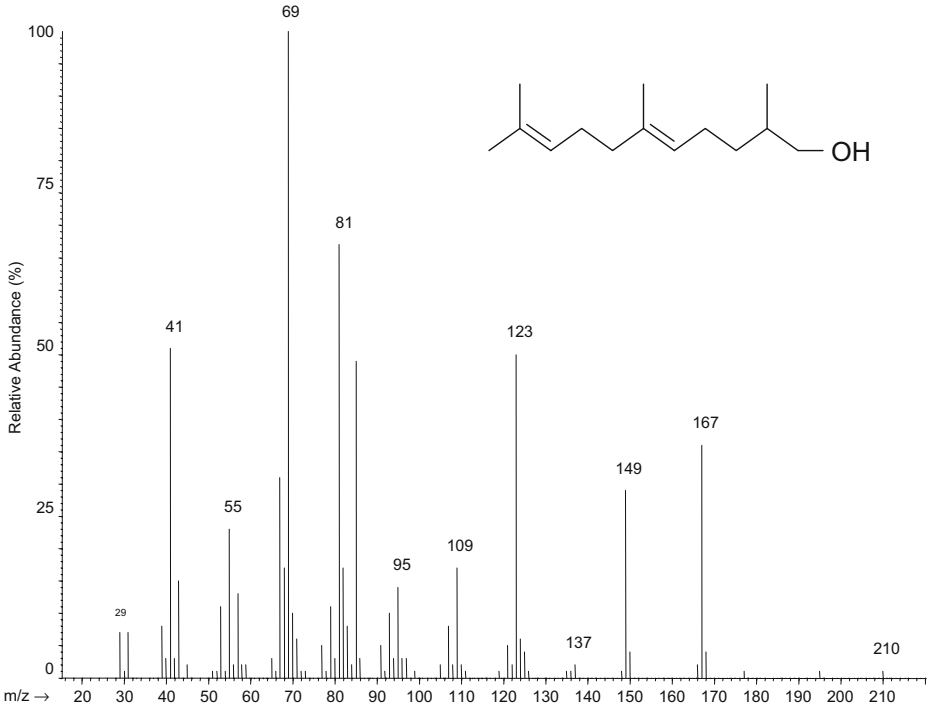


Fig. 3 EI mass spectrum of the compound specific to the sternal gland surface, identified as (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol

with the (*Z*)-isomer for the threshold activity, but higher concentrations of this isomer did not induce a reduction in trail-following. Thus, with the (*Z*)-isomer, we did not obtain the classical bell-shaped curve of responses in relation to pheromone concentration. In addition, a mixture of the two isomers was more active than the (*E*)-isomer, the activity threshold being obtained from 10^{-2} ng/cm.

Table 6 Choice of trails made with synthetic (*E*)- and (*Z*)-isomers, and a mixture of both isomers of 2,6,10-trimethyl-5,9-undecadien-1-ol for *M. darwiniensis* (T-maze trail-following bioassays)

Concentration	(*E*) Isomer Hexane	(*Z*) Isomer Hexane	(*E*)+(*Z*) Isomers Hexane
10^{-3} ng/cm	54/36 NS (n=90)	57/33 NS (n=90)	26/34 NS (n=60)
10^{-2} ng/cm	57/33 NS (n=90)	51/39 NS (n=90)	48/12 S (n=60)
10^{-1} ng/cm	57/33 NS (n=90)	51/39 NS (n=90)	46/14 S (n=60)
1 ng/cm	115/25 S (n=150)	81/9 S (n=90)	48/12 S (n=60)
10 ng/cm	69/21 S (n=90)	69/21 S (n=90)	54/6 S (n=60)
100 ng/cm	38/22 NS (n=60)	48/12 S (n=60)	48/12 S (n=60)
1,000 ng/cm	34/26 NS (n=60)	–	–

S, result statistically significant; *NS*, result statistically non significant

Porotermes adamsoni For this species, the isomers were tested in open-field bioassays (Fig. 4). With the (*E*)-isomer, we obtained an activity threshold at 10^{-2} ng/cm, optimum trail-following at 10^{-1} ng/cm, a reduction in trail-following beyond 1 ng/cm, and no response at 1 μg/cm. With the (*Z*)-isomer, the activity threshold was only reached at a concentration of 1 ng/cm and a concentration of 100 ng/cm elicited extensive trail-following. A mixture of both isomers elicited trail-following similar to that obtained with the (*E*)-isomer alone, with an activity threshold at 10^{-2} ng/cm, optimal trail-following between 10^{-1} and 1 ng/cm, and a reduction in trail-following beyond 10 ng/cm.

Stolotermes victoriensis The activity of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol could not be tested with this species, due to the death of the colony maintained in France.

Quantification of (*E*)-2,6,10-Trimethyl-5,9-undecadien-1-ol in Sternal Gland Extracts

For *M. darwiniensis*, the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was estimated to be about 20 pg per individual. This was confirmed with bioassays: artificial trails made from five sternal glands/cm were more active than trails of 10 pg of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol/cm, as active as those of 100 pg/cm and less active than those of 1 ng/cm (Table 7).

For *P. adamsoni*, the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was estimated by GC analysis to be about 700 pg/individual, 35 times more than in *M. darwiniensis*. Bioassays confirmed this result. Trails made of five sternal glands/cm were more active than trails of 1 ng of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol/cm, as active as trails of 3.5 ng/cm, and less active than those of 10 ng/cm (Table 7).

For *S. victoriensis*, the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was estimated to be 4 pg/individual, five times less than in *M. darwiniensis* and 175 times less than in *P. adamsoni*. This was confirmed by trail-following bioassays. Pseudergates of *S. victoriensis* were not able to distinguish their own trails of 5×10^{-1} sternal gland/cm from

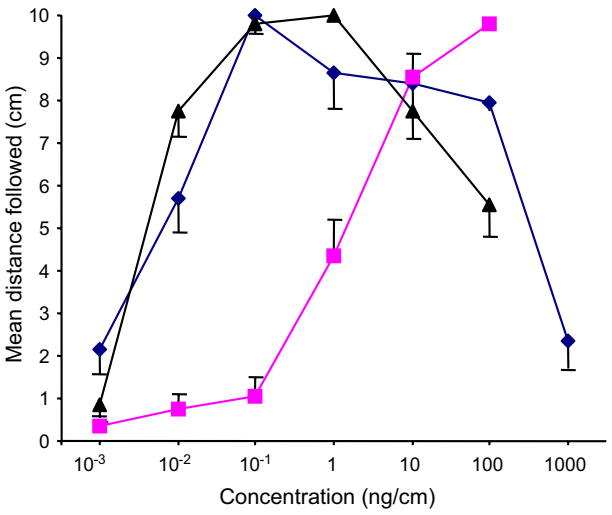


Fig. 4 Trail-following activity of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol (—◆—), (*Z*)-2,6,10-trimethyl-5,9-undecadien-1-ol (—■—), and a mixture of both isomers (—▲—) for pseudergates of *P. adamsoni* ('open-field' trail-following bioassay, *N*=30)

Table 7 Comparison of the trail-following activity of trails made from sternal gland extracts (*M. darwiniensis* or *P. adamsoni*), and synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol

Trail	Sternal Gland/cm	(*E*)-2,6,10—Trimethyl-5,9—undecadien-1-ol (ng/cm)					χ^2 test
Trail concentration	5	10^{-2}	10^{-1}	1	3.5	10	
Number of choices for *M. darwiniensis*	28	2	–	–	–	–	S
	11	–	19	–	–	–	NS
	7	–	–	23	–	–	S
Number of choices for *P. adamsoni*	24	–	–	6	–	–	S
	17	–	–	–	13	–	NS
	4	–	–	–	–	26	S

Results confirm the quantitative estimation of about 20 pg of pheromone per worker in *M. darwiniensis*, and 700 pg of pheromone per pseudergate in *P. adamsoni*

S, result statistically significant; *NS*, result statistically non significant; $N=30$

trails of 10^{-1} sternal gland of *M. darwiniensis*/cm (17 choices for the *S. victoriensis* trail, 13 choices for the *M. darwiniensis* trail, $N=30$).

Whereas the quantity of trail pheromone in individual sternal glands of *P. adamsoni* was 175 times greater than in *S. victoriensis*, it was not possible in the present study to evaluate the quantity of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol in their natural trails by direct measurement.

Discussion

The major roles of the trail-following pheromones in termites are recruitment and orientation. For example, in species of the 'one-piece life type', the pheromone alerts and recruits termites to repair a breach in the nest; in species of the 'separate life type', it also induces recruitment and orientation of foragers outside the nest (Traniello 1982; Hall and Traniello 1985; Traniello and Leuthold 2000). However, we have shown for 'separate life type' species (Pasteels and Bordereau 1998; Wobst et al. 1999; Peppuy et al. 2001a, b) and in this study for the 'one piece' species *P. adamsoni* that both ecological types were able to orientate on artificial trails in an open field experiment. Under the same experimental conditions, the situation was shown to be different for *M. darwiniensis*, which showed no tendency to follow open field trails, independent of the solvents used for extracting the pheromone or the concentration of the trail. Furthermore, whereas pseudergates of *P. adamsoni* form single files of individuals to explore a new environment, just as do workers of 'separate life type' species, workers of *M. darwiniensis* explore the same new environment individually or in tandem. Therefore, the first aim of our study was to know whether the most ancestral extant termite, *M. darwiniensis*, secreted a trail-following pheromone.

Our observations showed that workers of *M. darwiniensis* use a trail-following pheromone secreted from their sternal glands. However, this termite remains the only known species in which a trail pheromone alone does not elicit orientation. Thus, in addition to a trail pheromone, this termite requires a physical constraint, such as a tunnel or gallery, to guide its movement. The question then arose as to whether this unique situation was due to the intrinsic behavior-eliciting properties of the pheromone or to other specific termite behavior. Results obtained with *P. adamsoni* showed that the nature and the

concentration of the pheromone were not responsible for this special behavioral trait of *M. darwiniensis*. Thus, *P. adamsoni* used the same trail pheromone as *M. darwiniensis*, and it was readily able to follow artificial trails made from sternal glands of *M. darwiniensis* in open field assays, whereas the same trails were not followed by workers of *M. darwiniensis*. The unique exploratory behavior of *M. darwiniensis* workers that forage individually or in tandem but not in single file might represent a specific stage in the evolution of the recruitment strategies in termites. Indeed, according to Traniello and Leuthold (2000), mass recruitment might have evolved in social insects from a communication of tandem running type. The ancestral function of the sternal gland in termites, that is the secretion of semiochemicals that mediate the tandem running of swarming imagos at the time of the nuptial promenade, would have evolved in derived species to induce mass recruitment and orientation of foragers. The unusual formation of worker tandems as observed in *M. darwiniensis* during exploratory behavior, therefore, supports this evolution.

Our observations also highlight the uniqueness of *M. darwiniensis* regarding the glandular source of its trail pheromone. Whereas for all other termite species the trail pheromone is secreted by only one sternal gland, it is secreted by at least two sternal glands (sternal glands 3 and 4) in *M. darwiniensis*, which is the only termite known to possess three sternal glands (Noirot 1969). Extracts of sternal gland 4 were much more active than those from sternal gland 3, whereas extracts from sternal gland 5 had minimal activity. Therefore, *M. darwiniensis* might represent an intermediary taxon in a regressive evolution from lost ancestors with numerous metameric glands to the present species of termites that possess only one sternal gland located on sternite 4 or 5. The higher secretory activity of sternal gland 4 of *M. darwiniensis* is significant, given the position of the sternal gland on sternite 4 in the other basal termite families Termopsidae and Hodotermitidae.

The identification of the trail-following pheromone of the ancestral termites *M. darwiniensis, P. adamsoni,* and *S. victoriensis* was the second major aim of our study. We first showed that these three species secreted the same trail pheromone, which was identified as the norsesquiterpene alcohol (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol. This is a new structure for termite trail pheromones. Until now, only unsaturated C12 aliphatic alcohols and a C20 hydrocarbon have been identified as termite trail pheromones: (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol in numerous Rhinotermitidae and Termitidae (Matsumura et al. 1968; Tokoro et al. 1989, 1991; Laduguie et al. 1994; Pasteels and Bordereau 1998; Wobst et al. 1999), (3*Z*,6*Z*)-3,6-dodecadien-1-ol in *Ancistrotermes pakistanicus* (Termitidae Macrotermitinae; Robert et al. 2004), (*Z*)-3-dodecen-1-ol in several Termitidae (Peppuy et al. 2001a, b), and the diterpene hydrocarbon neocembrene in the Rhinotermitidae, Prorhinotermitinae (Sillam-Dussès et al. 2006), and in the Termitidae, Nasutitermitinae (Birch et al. 1972; MacDowell and Oloo 1984).

(*E*)-2,6,10-Trimethyl-5,9-undecadien-1-ol is known in the perfumery industry for its aromas of lemon and lily of the valley (Gora and Antczak 1980) but has never been identified previously in animals. However, an isomer of this compound, (*E*)-4,6,8-trimethyl-7,9-undecadien-5-ol, is secreted from sexual glands of female *Cryptocercus punctulatus* (Le Quéré et al. 1991) and *C. kyebangensis* (Grandcolas et al. 2001), subsocial cockroaches considered as sister taxa of *M. darwiniensis* (Eggleton 2001; Lo et al. 2003). Furthermore, a similar compound, 3,7,11-trimethyl-6,10-dodecadien-1-ol, is secreted by numerous species of bumblebees as a signal sex pheromone, and ants as a trail-marking pheromone (Francke and Schulz 1999; Luxova et al. 2004).

The biosynthesis of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol probably arises from mevalonate or farnesyldiphosphate (Prestwich 1981; Luxova et al. 2004; Hojo et al. 2007), whereas C12 alcohol pheromones probably arise from fatty acid biosynthesis,

especially from linoleic acid or oleic acid (Francke and Schulz 1999; Morgan 2004; Robert et al. 2004). The trail-following pheromone of *M. darwiniensis* and the Termopsidae *P. adamsoni* and *S. victoriensis* therefore represents a new chemical class of pheromones in termites.

The question arose as to whether (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was the sole compound of the trail pheromone or only its major compound. The pheromones of insects are generally composed of blends, and several observations suggested the existence of multicomponent pheromones in termites (Kaib et al. 1982; Traniello 1982; Hall and Traniello 1985; Pasteels and Bordereau 1998; Traniello and Leuthold 2000; Peppuy et al. 2001a, b). In *M. darwiniensis* and *P. adamsoni*, (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was the only specific compound detected from the surface of the sternal glands by SPME analysis. The GC fraction containing only (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was also the only fraction that elicited trail-following. These data suggested that the trail pheromone of *M darwiniensis, P. adamsoni*, and *S. victoriensis*, is comprised solely of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol. Nevertheless, trail-following bioassays performed at high concentrations showed that the GC fraction following that of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol and containing cuticular hydrocarbons also elicited trail-following, although to a lesser degree. This could be explained by the presence of an additional undetected component of the trail pheromone, but not by the presence of the (Z)-isomer of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol that is more volatile than the (*E*)-isomer. We also demonstrated that cuticular hydrocarbons from workers were inactive in eliciting trail-following in *M. darwiniensis*. More likely, it is the result of a tailing peak of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol, as has been observed previously for dodecatrienol or dodecenol after preparative GC in termite species that secrete these compounds as a trail pheromone (Peppuy et al. 2001a; Bordereau, unpublished results).

For *M. darwiniensis*, the activity threshold of synthetic (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol was obtained from 1 ng/cm of trail, i.e., in the high value range known for the activity of termite trail pheromones (Pasteels and Bordereau 1998; Sillam-Dussès 2004). However, this threshold might be dependent on the absolute configuration of the compound, as has been demonstrated previously in insects (Mori 1998) and especially in bumblebees for 3,7,11-trimethyl-6,10-dodecadien-1-ol (Luxova et al. 2004). The chirality of (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol in *M. darwiniensis* has not yet been determined. For *P. adamsoni*, (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol elicited trail-following from 10^{-2} ng/cm of trail, a threshold typical of many other termite trail pheromones, for example with dodecenol in Macrotermitinae (Peppuy et al. 2001a; Sillam-Dussès 2004).

The third contribution of our study concerns phylogeny. Although chemical communication in termites appears to be highly conserved (Pasteels and Bordereau 1998; Sillam-Dussès 2004; Sillam-Dussès et al. 2006), our study on trail pheromones of ancestral termites highlights some novel phylogenetic data. First, female adults of the subsocial cockroach *Cryptocercus*, a sister taxon of *M. darwiniensis* (Inward et al. 2007), secrete an isomer of the trail pheromone of *M. darwiniensis* from their tergal glands. This would support the phylogenetic relationships between cockroaches and termites, although it also could be a simple phenomenon of convergence because these isomers might arise from different metabolic pathways. Second, whereas the position of the Termopsidae remains controversial from morphological and molecular phylogenetic studies (Eggleton 2001), we showed that the only extant representative of the Mastotermitidae, *M. darwiniensis*, secreted the same trail pheromone as the representatives of two subfamilies of the Termopsidae (Porotermitinae and Stolotermitinae). This supports a basal position of Termopsidae (Thompson et al. 2000) and that the Termopsidae would be socially the most

primitive termites (Thorne and Carpenter 1992; Thorne 1997; Thorne and Traniello 2003). Our results also confirmed the basal position of Stolotermitinae, in contrast to the recent morphological study of Klass et al. (2000) that suggested relationships between Stolotermitinae and Kalotermitidae, Rhinotermitidae and Termitidae. Moreover, our study indicates a heterogeneity within the Termopsidae with the Porotermitinae and the Stolotermitinae in one group and the Termopsinae in the other. Thus, the Termopsidae might not be monophyletic.

The chemical nature of trail pheromones of termites separates the basal termites Mastotermitidae, Termopsidae, and Hodotermitidae with C14 or C18 trail pheromones from the other families (Kalotermitidae, Serritermitidae, Rhinotermitidae and Termitidae) that secrete C12 or C20 trail pheromones (Sillam-Dussès 2004; Sillam-Dussès et al. 2006). This aspect deserves to be studied in other taxa.

Acknowledgement CB is grateful to Theodore Evans, Sil Ruenko, Patrick Gleeson (CSIRO Entomology, Canberra), Michael Neal, Brian Thistleton (Department of Primary Industries, Darwin), and John Holt (James Cook University, Townsville) for hospitality, assistance with termite collections, and discussions on *M. darwiniensis* biology. He also thanks Daniel Fouquet (CIRAD, Montpellier, France) for the gift of the *M. darwiniensis* colony initially established in 1973 at the BAM (Berlin, Germany). The authors thank the Australian authorities for the export permits Nos. PWS P20012196 and WT 2002-29397.

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Introduction

The box tree pyralid, *Glyphodes perspectalis*, (Lepidoptera: Crambidae) is distributed in much of Asia, including throughout Japan, Korea, China, and India (Inoue et al. 1982). In Japan, the seriousness of damage to box trees, *Buxus microphylla* Sieb. et Zucc., caused by this pest has increased considerably (Kamizumi 1975; Maruyama and Shinkaji 1987, 1991, 1993; Maruyama 1992, 1993). Insecticide application has been used to control the box tree pyralid in parks and tree-lined streets in Japan. However, such control is complicated by the multiple generations of this pest that occur each year. For example, the Tokyo-Chiba population has three generations per year, with the adults appearing from mid-May to late June, from late July to late August and from late August to mid-September (Maruyama and Shinkaji 1987). If a synthetic pheromone for this insect were identified, it could be used to time insecticide applications more effectively, or it could be used as a possible control agent (e.g., mating disruption) by itself. We, therefore, undertook this study to identify the sex pheromone of this species.

Methods and Materials

Insect G. perspectalis larvae were collected at RIKEN, Saitama Prefecture, Japan, in 2005 and reared on box tree leaves. The insects were kept at 25°C under a 16:8 photoperiod. After emergence, females and males were separated and provided with 5% honey solution as food.

Preparation of Pheromone Extracts Ovipositor tips were excised with a pair of micro-scissors from 2- to 6-d-old virgin female moths, 3–6 hr into the scotophase. The tips were extracted in hexane for 15 min at room temperature. Extracts were placed in screw cap vials and stored at −20°C until analysis.

Chemicals Authentic (*Z*)-11-hexadecenal (Z11-16:Ald), (*E*)-11-hexadecenal (E11-16:Ald), and (*Z*)-11-hexadecen-1-ol (Z11-16:OH) were obtained from Pherobank (Wageningen, The Netherlands). Gas chromatography (GC) analyses showed these compounds to contain <0.1% of the corresponding geometric isomers.

Gas Chromatography GC analysis was conducted by using a DB-23 (30 m×0.25 mm ID, J&W Scientific, Folsom, CA, USA) and an HP-5MS (25 m×0.25 mm ID, Yokokawa Hewlett-Packard, Tokyo, Japan) column with a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (FID) and a splitless injector system. Helium was used as the carrier gas. The DB-23 column temperature was set initially at 100°C for 2 min and then increased to 250°C at 5°C min^{-1}; the temperature of the detector and injector was 250°C. The HP-5MS column temperature was initially set at 150°C for 2 min and then increased to 260°C at 10°C min^{-1}.

Gas Chromatograph-Electroantennographic Detection Gas chromatograph-electroantennographic detection (GC-EAD) analysis was performed by using an Hewlett-Packard 5890 Series II GC equipped with an HP-5MS column. The GC had splitless injection and used helium as carrier gas. The oven temperature was held at 100°C for 2 min and then increased to 250°C at 5°C min^{-1}. The effluent from the column was split at a 1:1 ratio between the FID and

EAD. Humidified air (21°C) delivered the GC effluent to the antennal preparation that was connected to an amplifier via Ag–AgCl electrodes immersed in 0.1 M KCl.

Gas Chromatography-Mass spectrometry Gas chromatography-mass spectrometry (GC-MS) analyses were conducted with a Hewlett-Packard 5972 MSD coupled to a Hewlett-Packard 5890 series II gas chromatograph equipped with splitless injection and an HP-5MS column. The column oven temperature was held at 100°C for 2 min and then increased to 240°C at a rate of 10°C min^{-1}. Electron ionization MS was conducted at 70 eV.

Dimethyl Disulfide Derivatization Reaction of monounsaturated components with dimethyl disulfide (DMDS) was performed to determine double bond positions. Crude extracts were dissolved in 50 μl of hexane, and added to 50 μl of DMDS and 10 μl of iodine solution (60 mg in 1 ml of ether). The reaction was carried out in Teflon-lined screw vials, overnight at 40°C. After this, 200 μl of hexane, and 50 μl of $Na_2S_2O_3$ solution (5% in distilled water) were added. The extracts were dried with anhydrous Na_2SO_4 and concentrated for GC-MS analysis.

Laboratory Bioassays Laboratory bioassays were conducted in a screen cage (32×40×30 cm) under the same conditions as described above. A filter paper strip (2×4 cm), impregnated with 2 μl of a hexane solution containing a synthetic pheromone blend, was suspended 5 cm below the center of the top screen. Ten virgin males, 2–6 d after emergence, were released into the cage 3–6 hr into the scotophase. The total number of contacts of males, claspers extruded, with the treated filter paper was recorded for 5 min. Each bioassay was replicated four to ten times. The blend ratio of, Z11-16:Ald, E11-16:Ald, and Z11-16:OH in the treatments was 5:1.25:1, and the amount of Z11-16:Ald 100 ng/female. The differences among the mean numbers of contacts of males per replicate were tested by a Tukey–Kramer test. Significant differences are reported at $P<0.05$.

Field Bioassays Field bioassays were conducted in Box tree, *B. microphylla*, fields at RIKEN, Saitama Prefecture, Japan, May 20–August 20, 2006. Sticky traps (Sumika-Takeda Agrochemical Co., Ltd., Tokyo, Japan) were baited with grey halo-butyl isoprene blend elastomer septa (West Company, Singapore), which were renewed every month. Two traps of each treatment were used, with approximately 10 m as the distance between traps. The blend ratio of compounds, Z11-16:Ald, E11-16:Ald, and Z11-16:OH tested was 5:1.25:1, and the amount of Z11-16:Ald was 0.5 mg/septum. Traps were checked every 10 d for numbers of males. After checking, the sticky inserts were renewed, and trap positions moved. For statistical analysis, the number of males caught at each trap at each time of checking was transformed to $(X+0.5)^{1/2}$, and the differences among the means of catches each check (i.e., every 10 d) were tested by a Tukey–Kramer test. Significant differences are reported at $P<0.05$.

Results

GC-EAD Two major peaks were observed on the EAD trace of ovipositor extract of virgin *G. perspectalis*,; these corresponded with peaks (designated A and B) on the FID chromatogram (Fig. 1).

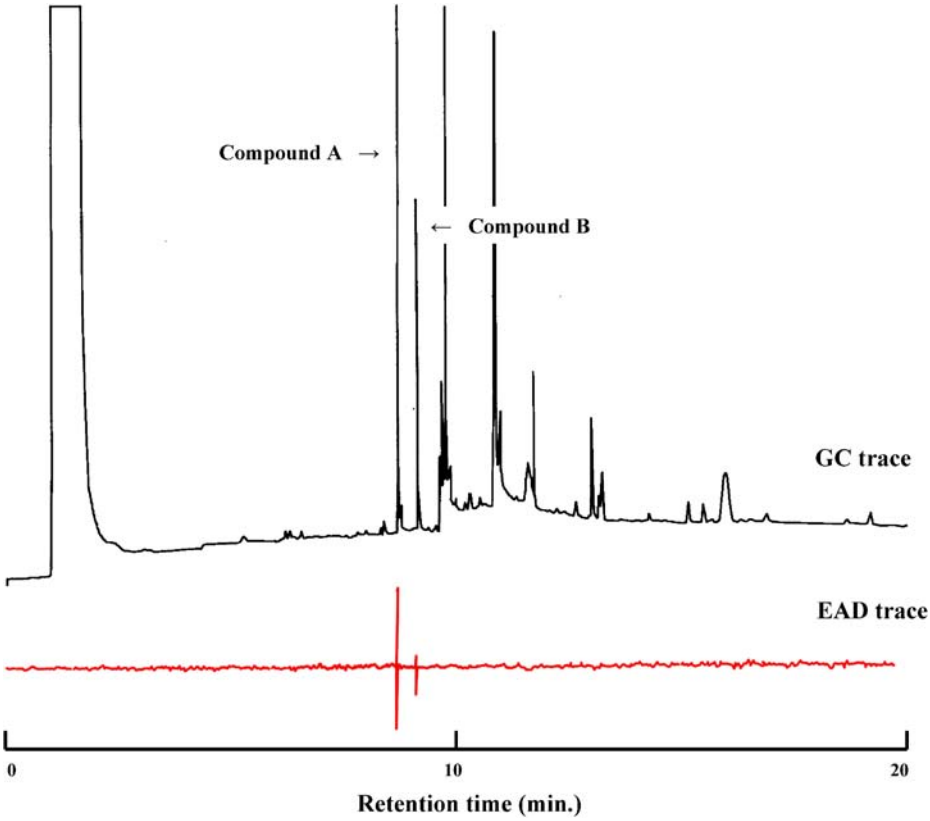


Fig. 1 Gas chromatograph-electroantennogram detection analysis of crude ovipositor extract of virgin *G. perspectalis* females using a male antenna. Compound A = (*Z*)- and (*E*)-11-hexadecenal, Compound B = (*Z*)-11-hexadecenol

Chemical Analyses GC-MS analysis of the crude extracts on the nonpolar HP-5MS column showed Peak A to have a mass spectrum characteristic of hexadecenal, containing *m/z* 238 (M^+, 1.0%), *m/z* 220 (M^+-18, 4.2 %), and *m/z* 55 (base peak, 100%). Peak B gave a mass spectrum characteristic of 11-hexadecen-1-ol, containing *m/z* 222 (M^+-18, 3.4%) and *m/z* 55 (base peak, 100%).

Mass spectral analysis of the DMDS reaction products showed the presence of two DMDS adducts (*m/z* 332) of hexadecenal derivatives. The mass spectrum indicated that both adducts had a double bond at the 11-position, based on fragment ions at *m/z* 332 (M^+), *m/z* 215 (A^+), *m/z* 117 (B^+), and *m/z* 61 (base peak). GC-MS analysis of the DMDS reaction products also showed the presence of an 11-hexadecen-1-ol derivative with a double bond at the 11-position, based on *m/z* 334 (M^+), *m/z* 217 (A^+, base peak), and *m/z* 117 (B^+).

The retention times of the three compounds on the polar DB-23 column coincided with those of synthetic Z11-16:Ald (13.73 min), E11-16:Ald (13.46 min), and Z11-16:OH (15.71 min). Similarly, on the nonpolar HP-5MS column, the retention times of the two peaks coincided with those of synthetic Z11-16:Ald (8.89 min), E11-16:Ald (8.89 min), and Z11-16:OH (9.82 min). The three compounds were, therefore, identified as Z11-16:Ald,

E11-16:Ald, and Z11-16:OH. The ratio of these three compounds in the crude extract was roughly 5:1.25:1, respectively, and the total amount approximately 100 ng/female.

Laboratory Bioassays In the laboratory bioassay that tested the three compounds, only Z11-16:Ald elicited significant responses from males (Fig. 2). The level of response to this compound was not significantly different from the level of response to crude extract. The addition of E11-16:Ald to Z11-16:Ald, Z11-16:OH to Z11-16:Ald, or Z11-16:OH to a mixture of Z and E11-16:Ald, gave no significant change in response compared to that to Z11-16:Ald alone (Fig. 2).

Field Bioassay The mixture of Z and E11-16:Ald, at the natural ratio, gave trap catches that were significantly higher than those in control traps (Fig. 3). However, the major compound, Z11-16:Ald, alone was not attractive to males in the field. The addition of Z11-16:OH to the mixture of Z and E11-16:Ald resulted in a significant depression of trap catches relative to the two component blend.

Discussion

On the basis of GC-EAD, GC, and GC-MS analysis, as well as DMDS derivatization, three compounds were identified in the pheromone gland of female *G. perspectalis*, namely, Z11-16:Ald, E11-16:Ald, and Z11-16:OH. In laboratory bioassays, Z11-16:Ald alone showed significant attractiveness to males. However, in field trials, this compound alone baited in traps did not elicit catches of male *G. perspectalis*. Instead a mixture of Z11-16:Ald and E11-16:Ald was required for significant trap catches (relative to the control). Furthermore, addition of Z11-16:OH to the two component blend resulted in a decreased trap catch of males, but in

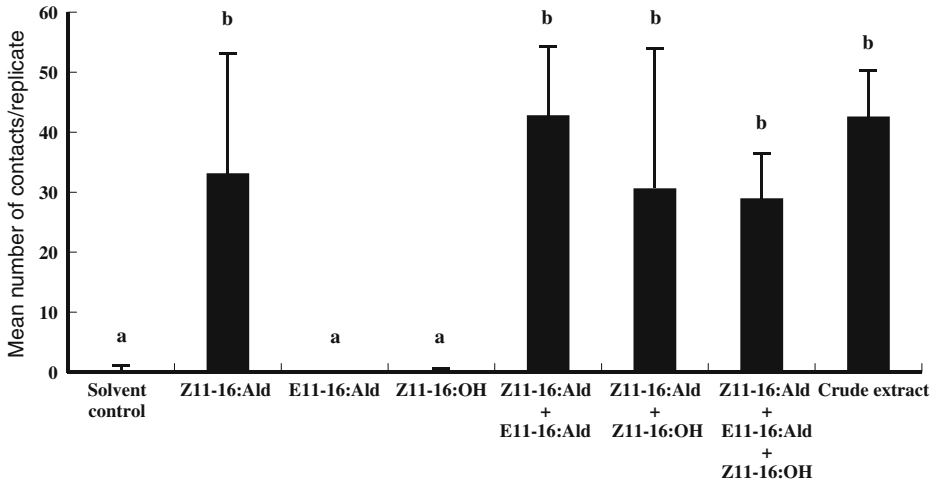


Fig. 2 Laboratory bioassay testing the responses (mean number of contacts/replicate) of male *G. perspectalis* to various treatments. Each treatment, consisting of ten males, was replicated four to ten times. Means with the same letter are not significantly different at $P<0.05$ (Tukey–Kramer test). *Bars* represent standard error of the means

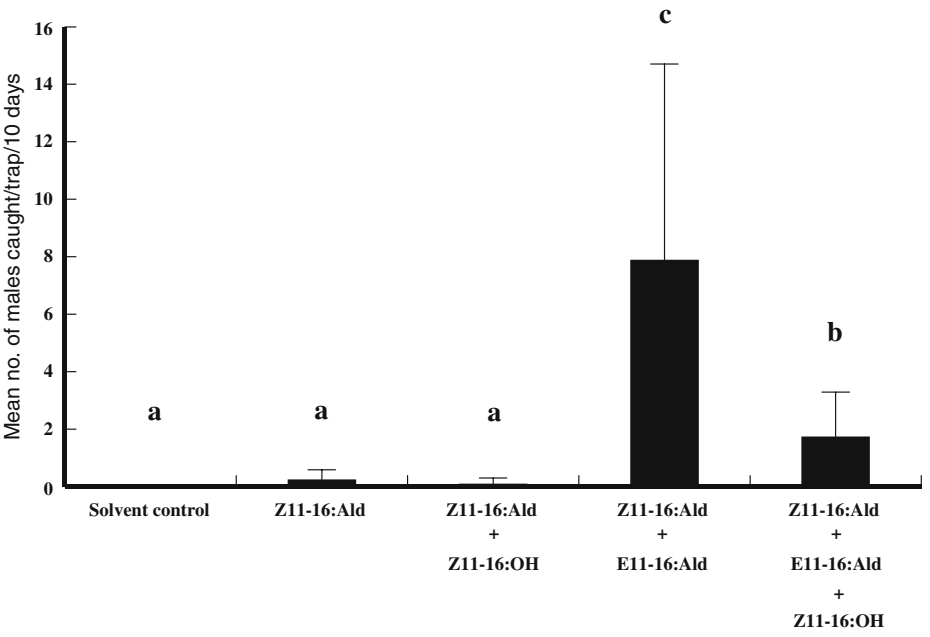


Fig. 3 Field catches of male *G. perspectalis* in traps baited with various chemical treatments. *Values followed by the same letter* are not significantly different at $P<0.05$ (Tukey–Kramer test). *Bars* represent standard error of the means

the laboratory bioassay, no antagonistic effect of the alcohol was found. These inconsistencies between the field and laboratory bioassays are likely due to the limited flight space in the cage not requiring males to maintain sustained upwind flight after activation by the respective blends.

Based on the field and laboratory bioassays, we conclude that the blend of Z11-16:Ald and E11-16:Ald in a ratio of roughly 5:1.25 comprises an attractive sex pheromone blend for *G. perspectalis*. Given the antagonistic effect of Z11-16:OH in the field trials, it seems likely that this compound is a biochemical precursor of the major component Z11-16:Ald (Bjostad et al. 1987; Jurenka and Roelofs 1993) and is not actually released by female *G. perspectalis*.

It is known that eight species of Crambidae utilize Z11-16:Ald as a sex pheromone component, including *Agriphila straminella* (Booij and Voerman 1984), *Catoptria permutatella* (Booij and Voerman 1984), *Chilo partellus* (Nesbitt et al. 1979; Hansson et al. 1995), *Chilo suppressalis* (Nesbitt et al. 1975; Tatsuki et al. 1983), *Chrysoteuchia topiaria* (Kamm and Mcdonough 1980), *Diatraea grandiosella* (Hedin et al. 1986), *Parapediasia teterrella* (Clark and Haynes 1990), and *Tehama bonifatella* (McDonough et al. 1982). However, none of these species utilize the opposite geometric isomer (E11-16:Ald), found in *G. perspectalis*, but rather utilize a positional isomer as a minor component. In another *Glyphodes* species, the mulberry pyralid, *G. pyloalis*, the major component of the female-produced sex pheromone has been identified as (*E, E, Z*)-10,12,14-hexadecatrienyl acetate (Seol et al. 1987, Honda et al. 1990). It is well-established that taxonomically related species of moths may utilize common sex pheromone components (Tamaki 1976). However, this trienic compound appears to have no direct structural relationship to the *G. perspectalis* compounds, except for the length of the carbon chain, even though the two

species are congeners. Studies on the sex pheromones of other *Glyphodes* species are warranted to determine what compounds, if any, are common in this genus.

Acknowledgements We are grateful to Sankei Chemical Co., Ltd. (Tokyo, Japan) and West Company (Singapore, Republic of Singapore) for supplying the rubber septa.

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Planococcus minor and the closely related mealybug, *Planococcus citri* (Risso), are similar morphologically (Venette and Davis 2004). In a study that tested the synthetic sex pheromone of *P. citri* (Bierl-Leonhardt et al. 1981) in Taiwan, Tu et al. (1988) reported that some mealybugs thought to be *P. citri* were not attracted to synthetic sex pheromone. These unresponsive mealybugs were later identified as *P. minor* (Tu et al. 1988).

The sex pheromones of two *Planococcus* species, *P. citri* and *Planococcus ficus* Signoret, have been identified and are used for management of these pests (Moreno et al. 1984; Millar et al. 2002; Walton et al. 2006). Considering the importance of *P. minor* as a pest, identification of its sex pheromone would also be useful. We report in this paper the identification of the sex pheromone of *P. minor* as (*E*)-2-isopropyl-5-methyl-2,4-hexadienyl acetate.

Methods and Materials

Insects Mealybugs, originally collected from star fruit (*Averhoa carambola* L.) in the central area of Taiwan, were reared on pumpkins, *Cucurbita moschata* Duch. Poiret, at 25±1°C, 70±5% relative humidity under a photoperiod of 12L:12D. The identity of the species was confirmed by Dr. Doug Miller and colleagues at the United States Department of Agriculture in Beltsville, Maryland, USA.

Pumpkins infested with ovisacs (60–70 ovisacs per pumpkin) were placed on a shallow plastic pan in a walk-in incubator. After 16 to 20 d, male nymphs of *P. minor* were separated by the method of Negishi et al. (1980) and Arai (2000). Briefly, male nymphs were allowed to crawl off the pumpkins and pupate on paper towels that covered the pumpkins. The paper towels were then placed in a container (21.5 cm ID.×6.5 cm in depth) in an incubator, and adult males allowed to emerge. Adult males, 2–3 d after emergence, were used in bioassays. Virgin females, after the last molt, were transferred to a new pumpkin with a fine paintbrush so as to prevent any mating with males that had not moved onto the paper towels. Each pumpkin was infested with about 1000–2000 females. Females were used for collection of pheromone 5–7 d after the final molt.

Collection of Pheromone Extract Pumpkins infested with virgin females were placed in a 30×30×30 cm^3 chamber, with purified air passing through the chamber. Because the males' responses to females were strongest during the first 2 hr of the photophase (Hwang and Chu 1987), the headspace volatiles were collected for the first 4 hr of the photophase on a tube (2.5 cm×8 mm ID) of Porapak-Q held in place with glass wool plugs. A total of 10,000–40,000 female-day equivalents (hereafter, FDE) of sex pheromone was collected in each tube. Aerations of uninfested pumpkins served as controls. Chemicals adsorbed on the Porapak-Q were extracted with pentane.

Analysis of Pheromone Extracts Extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) with a ThermoQuest Trace GC interfaced to a Finnigan Trace mass spectrometer (electron impact ionization, 70 eV). The GC oven was held at 40°C for 1 min, then programmed at 10°C min^{-1} to 250°C. DB-5MS, DB-1, and DB-23 columns (all 30 m×0.25 mm ID, J & W Scientific, Folsom, CA, USA) were used for analyses. The injection was splitless, with helium as carrier gas. NMR spectra were recorded on Bruker Avance 300 and Bruker DRX500 spectrometers. High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-700 mass spectrometer.

Isolation of Pheromone High-performance liquid chromatography (HPLC) was employed to isolate the sex pheromone by using an ECOM® Beta 10 Gradient Pump (Prague, Czech Republic), a silica gel column (250×4.6 mm, 5 μm, LDC/Milton Roy, Riviera Beach, FL, USA), a Rheodyne Model 7125 injector, and a DAD230 photo diode array detector (set at 200–650 nm) (ChromTech, Singapore). Extracts from collections of infested and uninfested pumpkins were eluted with a solvent gradient of pentane and diethyl ether (1.0 ml/min), starting with 100% pentane for 5 min, and then decreasing to 85% pentane over 75 min with a concomitant increase of diethyl ether. Fractions were collected every minute with a fraction collector. Fractions were concentrated under a stream of nitrogen and analyzed by GC-MS.

Hydrolysis of the Unknown The fraction containing the unknown (ca. 1 μg) was dissolved in 300 μl of ethanol, and two drops of 1 N NaOH were added. The reaction mixture was kept in a water bath at 35°C for about 30 min before 1 N HCl was added to neutralize the solution. The product was extracted with hexane. The hexane solution was dried by passage through a short plug of anhydrous $MgSO_4$. After concentration under a stream of nitrogen, the product was analyzed by GC-MS.

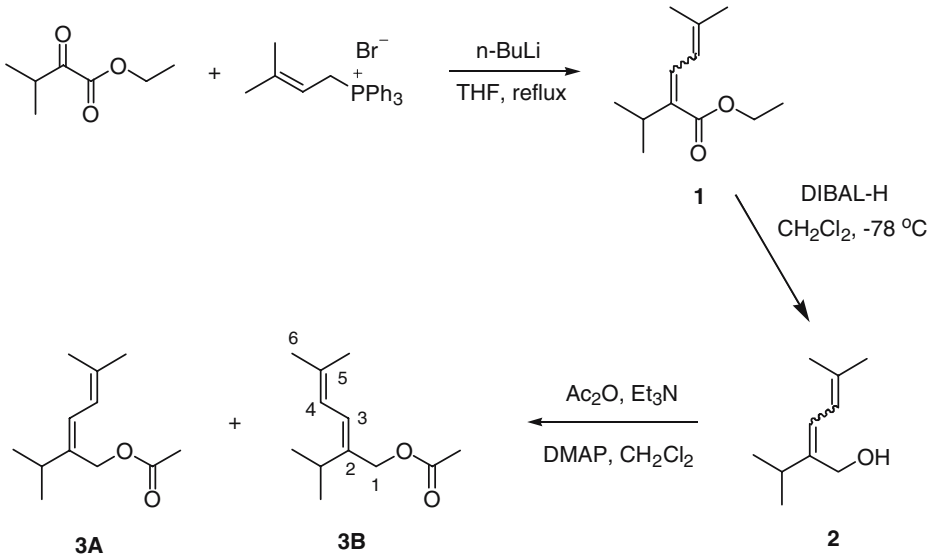
Hydrogenation of the Unknown One microgram of the isolated unknown was reduced in a septum-capped vial by catalytic reduction with 5% Pd on carbon and a balloon of H_2. After about 30 min, the reaction mixture was filtered through powdered $MgSO_4$ to remove the catalyst. The filtrate was analyzed by GC-MS.

Chemicals Triphenylphosphine, ethyl 3-methyl-2-oxobutyrate, 1-bromo-3-methyl-2-butene, *n*-butyl lithium (*n*-BuLi), tetrahydrofuran (THF), dimethylaminopyridine (DMAP), triethylamine, and acetic anhydride were obtained from Sigma-Aldrich Co. (Milwaukee, WI, USA). Diisobutylaluminum hydride (DIBAL-H) was from Strem Chemicals, Inc. (Newburyport, MA, USA), and 2-isopropyl-5-methylhexyl acetate was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Laboratory Bioassay A turntable olfactometer (Tashiro et al. 1969) was used with the modifications described below. The turntable was set up in a room with lighting of 940± 120 lx and temperature at 25±2°C, and rotated at 0.67 rpm to reduce any positional effects. Fifteen to 20 sticky traps (6×6 cm^2) were placed on the wheel of a 2-m-diameter turntable, with treatments or controls placed at the center of the sticky traps. Males to be used in bioassays were kept in the dark from 17:00 of the previous day until 09:00 the day of the bioassay, with bioassays carried out during the first 4 hr of the photophase. Males (300–500 for each replicate) were released from a glass dish (20 cm i.d.) in the center of the turntable. Pieces of Parafilm-wrapped pumpkins (1.5×1.5×1 cm), with only the pumpkin skin exposed, were put in a plastic tube (3 cm i.d.×1.5 cm deep) as neutral controls. Pumpkins wrapped in the same way as the neutral controls but infested with 20 females were used as positive controls.

Synthesis of 2-Isopropyl-5-methyl-2,4-hexadienyl acetate (3) The geometric isomers **3A** and **3B** were synthesized as shown in Scheme 1. Details of the procedure are described below.

Preparation of (3-methyl-2-butenyl)Triphenyl phosphonium Bromide A mixture of triphenylphosphine (2.62 g, 1 mmol) and 1-bromo-3-methyl-2-butene (1.49 g, 1 mmol) was



Scheme 1 Synthesis of geometric isomers **3A** and **3B**

refluxed overnight in dry toluene (30 ml) under N_2. After cooling, the precipitate was filtered and washed with toluene. The crystalline product was dried under vacuum to give the phosphonium salt (4.11 g, yield 100%) as a white solid: mp 236–237°C (mp 235–236°C, Armesto et al. 1995). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.26 (3H, d, J=4.2 Hz), 1.64 (3H, d, J=6.0 Hz), 4.62 (2H, m), 5.12 (1H, m), 7.88–7.64 (15H, m).

*Preparation of Ethyl 2-isopropyl-5-methyl-2,4-hexadienoate (***1***)* *n*-BuLi (1.6 M, 6.3 ml, 10 mmol) was added slowly to a solution of (3-methyl-2-butenyl)triphenylphosphonium bromide (4.11 g, 10 mmol) in a 40-ml dry THF under N_2 at 0°C, until the solution turned deep red. The reaction mixture was brought to reflux. Ethyl 3-methyl-2-oxobutyrate (1 g, 6.9 mmol in 10 ml of dry THF) was added slowly and the mixture refluxed for another 15 hr. Water (10 ml) was added to the cooled reaction mixture and then 10% aqueous NH_4Cl to neutralize the solution. The mixture was extracted with ether (20 ml×3), and the ether extracts dried with anhydrous $MgSO_4$, filtered, and concentrated. Purification of the residue on a silica gel column (hexane–CH_2Cl_2 3:1) afforded a 1.3:1 mixture of stereoisomers of ethyl 2-isopropyl-5-methyl-2,4-hexadienoate (**1**, 0.71 g, yield 72%) determined by ^{1}H NMR spectroscopy. ^{1}H NMR spectrum ($CDCl_3$, 300 MHz) gave two sets of closely related signals: δ 1.10 (7.8H, d, J=6.9 Hz), 1.20 (6H, d, J=6.9 Hz), 1.31 (3.9H, t, J=7.2 Hz), 1.33 (3H, t, J=7.2 Hz), 1.82 (3.9H, s), 1.84 (3.9H, s), 1.88 (3H, s) 1.89 (3H, s), 2.79 (1.3H, septet, J=6.9 Hz), 3.03 (1H, septet, J=6.9 Hz), 4.12–4.29 (4.6H, m), 6.21 (1H, d, J=12.0 Hz), 6.52 (1.3H, d, J=12.0 Hz), 6.56 (1.3H, d, J=12.0 Hz), 7.31 (1H, d, J=12.0 Hz). The ^{13}C NMR spectrum gave 24 resonances, as expected for the mixture of two geometric isomers: δ 14.0 (2×C), 17.8, 18.4, 20.8 (2×C), 21.8 (2×C), 26.4, 26.6, 27.0, 31.4, 59.6, 59.7, 120.1, 121.9, 129.3, 133.0, 134.1, 135.5, 140.6, 143.6, 167.9, and 168.3.

*Preparation of 2-Isopropyl-5-methyl-2,4-hexadienol (***2***)* DIBAL-H (20% in hexane, 3.6 ml, 4.0 mmol) was added to a solution of the 1.3:1 mixture of ester **1** (294 mg, 1.5 mmol) in CH_2Cl_2 (15 ml) at −78°C under N_2. The reaction mixture was stirred for 30 min and then quenched with −78°C MeOH. Celite (1 g) and $Na_2SO_4{\cdot}10H_2O$ (1 g) were added and the

suspension warmed to room temperature and stirred for another 2 hr (Takano et al. 2001). Filtration and concentration of the resulting mixture, followed by flash chromatography on silica gel (CH_2Cl_2), gave a 1.3:1 mixture of the geometric isomers of alcohol **2** (217 mg, 94%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.08 (13.8H, d, J=6.9 Hz), 1.78 (7.8H, s), 1.82 (6H, s), 2.49 (1.3H, septet, J=6.9 Hz), 2.99 (1H, septet, J=6.9 Hz), 4.16 (2H, s), 4.24 (2.6H, s), 6.23–6.07 (4.6H, m). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.1 (2×C), 21.4 (2×C), 21.9 (2×C), 26.4, 26.5, 28.1, 33.8, 59.4, 64.3, 119.9, 120.1, 121.1, 122.2, 135.7, 135.9, 143.2, 144.0.

*Preparation of 2-Isopropyl-5-methyl-2,4-hexadienyl acetate (***3A** *and* **3B***)* To a solution of compound **2** (123 mg, 0.8 mmol) in CH_2Cl_2 (10 ml) was added acetic anhydride (204 mg, 2.0 mmol), triethylamine (202 mg, 2.0 mmol), and 4-dimethylaminopyridine (1.3 mg, 0.01 mmol) at room temperature. The solution was stirred for 30 min and then quenched with water. The reaction mixture was extracted with CH_2Cl_2 (10 ml×3). Extracts were combined, dried over $MgSO_4$, filtered, and concentrated *in vacuo* (Takano et al. 2001). Purification on a silica gel column afforded compound **3** (139 mg, 89%) as a mixture of two isomers. The mixture was separated by HPLC, with the same gradient program as that used for isolation of pheromone from the aeration collections, collecting fractions every 0.25 min. Compound **3A** gave a ^{1}H NMR ($CDCl_3$, 300 MHz) spectrum as follows: δ1.07 (6H, d, J=6.9 Hz, 2-CH(CH_3)$_2$), 1.79 (3H, s, 5-CH_3), 1.82 (3H, s, H-6), 2.07 (3H, s, 1-OCOCH_3), 2.44 (1H, septet, J=6.9 Hz, 2-CH(CH$_3$)$_2$), 4.72 (2H, s, H-1), 6.08 (1H, d, J= 11.7 Hz, H-4), 6.29 (1H, d, J=11.7 Hz, H-3). ^{13}C NMR ($CDCl_3$, 75 MHz) of compound **3A**: δ18.2 (5-*C*H$_3$), 21.1 (1-OCO*C*H$_3$), 21.8 (2-CH(*C*H$_3$)$_2$), 26.5 (C-6), 33.8 (2-*C*H(CH$_3$)$_2$), 61.1 (C-1), 120.0 (C-4), 124.4 (C-3), 136.9 (C-5), 138.3 (C-2), 171.3 (1-O*C*OCH$_3$). MS (m/z; %): 196 (5, M$^+$), 121 (100); HRMS m/z 196.1462 [M]$^+$ (calculated for $C_{12}H_{20}O_2$ 196.1463); UV (CH_2Cl_2) λ_{max} (log ε) 248 (4.9) nm. Compound **3B** ^{1}H NMR ($CDCl_3$, 300 MHz): δ1.07 (6H, d, J=6.9 Hz, 2-CH(CH_3)$_2$), 1.79 (3H, s, 5-CH_3), 1.82 (3H, s, H-6), 2.07 (3H, s, 1-OCOCH$_3$), 3.01 (1H, septet, J=6.9 Hz, 2-CH(CH$_3$)$_2$), 4.60 (2H, s, H-1), 6.08 (1H, d, J= 11.7 Hz, H-4), 6.23 (1H, d, J=11.7 Hz, H-3). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.2 (5-*C*H$_3$), 21.2 (1-OCO*C*H$_3$ and 2-CH(*C*H$_3$)$_2$), 26.5 (C-6), 28.1 (2-*C*H(CH$_3$)$_2$), 66.6 (C-1), 119.8 (C-4), 125.1 (C-3), 137.3 (C-5), 137.6 (C-2), 170.9 (1-O*C*OCH$_3$). MS m/z (%): 196 (5, M$^+$), 121 (100); HRMS m/z 196.1460 [M]$^+$ (calculated for $C_{12}H_{20}O_2$ 196.1463); UV (CH_2Cl_2) λ_{max} (logε) 248 (4.4) nm. Neat compound 3 gave diagnostic IR absorptions at 1707 (C=O), 1637 cm^{-1} (C=C).

Results

Analysis Comparison of the extracts of volatiles from mealybug-infested and uninfested pumpkins revealed one significant insect-produced compound, which was isolated as a single component of the fraction eluting at 50 min by HPLC. The Kovats Indices (KI) of this unknown were 1687 on the DB-23 column, 1347 on the DB-1 column, and 1350 on the DB-5MS column. In the bioassay, a 100-ng sample of the isolated compound attracted a mean (N=6) of 25.3 male mealybugs, whereas 20 females attracted a mean (N=6) of 3.2 male mealybugs, suggesting that the unknown was active. The mass spectrum of the compound (Fig. 1) showed significant fragments at m/z (%): 43 (18), 69 (7), 91 (13), 93 (18), 105 (13), 121 (100), 123 (11), 136 (16), with a small molecular ion at 196 (5). The presence of significant peaks at m/z 43 ($CH_3C{\equiv}O^+$) and 136 (M^+-CH_3COOH) and the

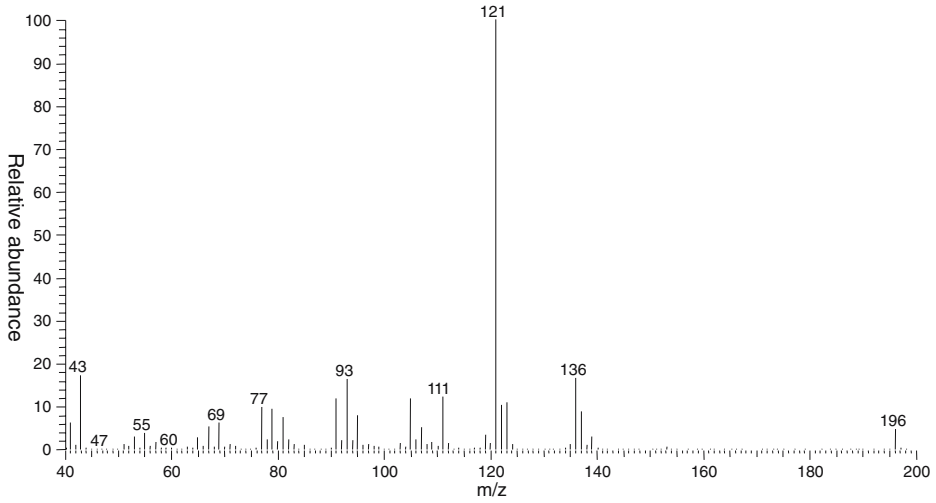


Fig. 1 EI mass spectrum (70 eV) of the active component in pheromone collections from female *Planococcus minor*

absence of other prominent fragmentations between *m*/*z* 196 and 136, suggested that the unknown was an acetate ester. After base hydrolysis, a compound with molecular weight *m*/*z* 154 was found in the reaction mixture. The mass spectrum (Fig. 2) of the hydrolyzed product showed fragment ions with *m*/*z* (%): 43 (34), 55 (38), 69 (34), 93 (46), 111 (100), and 123

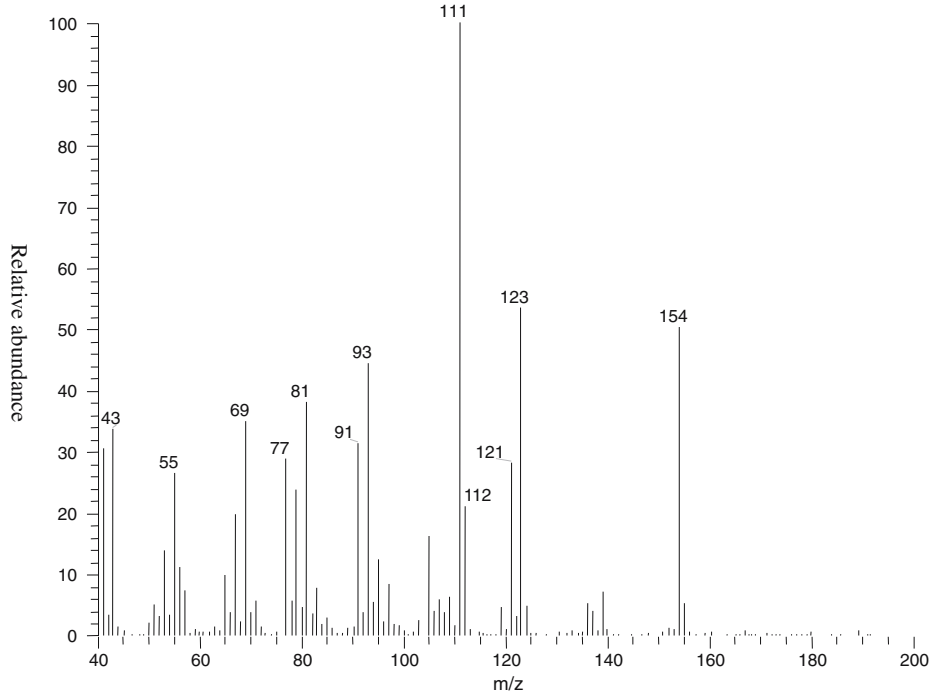


Fig. 2 EI mass spectrum (70 eV) of hydrolyzed active component in pheromone collections from female *Planococcus minor*

(54), and a molecular ion of 154 (50). The molecular weight of the hydrolyzed product was 42 mass units less than that of the unknown, supporting our hypothesis that the unknown was an acetate ester.

The mass spectrum (Fig. 3) of the hydrogenated product showed fragment ions with *m*/*z* (%): 43 (100), 61 (8), 69 (96), 84 (58), 97 (38), 112 (11), 140 (14), and 157 (6). Comparison of the mass spectrum of the hydrogenated product with the NIST mass spectral database suggested a possible structure for the hydrogenated product as 2-isopropyl-5-methylhexyl acetate. This was confirmed by comparing the GC retention time and the mass spectrum of the hydrogenated compound from the insect with that of an authentic standard. These results indicated that the unknown had a carbon skeleton of 2-isopropyl-5-methylhexyl acetate with two sites of unsaturation.

HPLC collection yielded about 3 μg of the unknown from 37,000 FDE of crude extract. The ^{1}H NMR spectrum of the unknown showed signals at δ 1.79 (3H, s), 1.82 (3H, s), 2.07 (3H, s), 4.60 (2H, s), 6.08 (1H, d, J=11.7 Hz), and 6.23 (1H, d, J=11.7 Hz). The six-proton singlet at δ 1.79 and 1.82 (two allylic methyl groups), typical acetyl protons (δ 2.07), oxygenated methylene protons (δ 4.60), and two vinylic protons mutually coupled with a coupling constant of 11.7 Hz, suggested a skeleton of 2-substituted-5-methyl-2,4-hexadienyl acetate. In addition, an isopropyl moiety was revealed by the signals at δ 1.07 (6H, d, J=6.9 Hz) and 3.01 (1H, septet, J=6.9 Hz). On the basis of the above, the unknown compound was identified as 2-isopropyl-5-methyl-2,4-hexadienyl acetate.

Synthesis The stereochemistry of the unknown compound was determined by a non-stereoselective synthesis followed by separation of the (*Z*)- and (*E*)-2-isopropyl-5-methyl-2,4-hexadienyl acetates (**3A** and **3B**). Thus, Wittig reaction between ethyl 3-methyl-2-

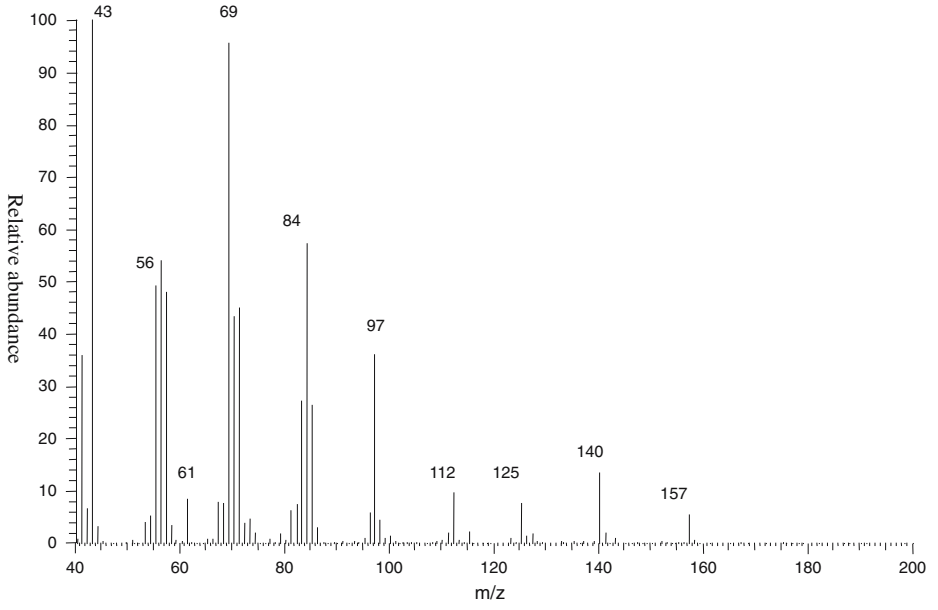


Fig. 3 EI mass spectrum (70 eV) of the hydrogenation product of the active component in pheromone collections from female *Planococcus minor*

oxobutyrate and the ylide from (3-methyl-2-butenyl)triphenylphosphonium bromide (formed by addition of *n*-BuLi) produced an isomeric mixture of **1** in a 1.3:1 ratio. GC-MS analysis of the mixture indicated that the two components had KIs of 1639 and 1651, respectively, on the DB-23 column. The two compounds exhibited similar mass fragmentation patterns, with fragments of *m/z* (%) 81 (28), 91 (21), 107 (49), 123 (100), 135 (12), 153 (25), 181 (28), and a molecular ion at 196 (41). Reduction of compound **1** with DIBAL-H yielded alcohols **2**, with KIs of 1691 and 1733, respectively, on the DB-23 column. The two isomers gave similar mass fragmentation patterns, with *m/z* (%) of 69 (40), 81 (34), 91 (36), 93 (42), 111 (100), 125 (55), and 154 (M^+, 53). The mixture was acetylated to produce the acetates **3** as a (*Z*)- and (*E*)- mixture with KIs of 1666 and 1687, respectively, on the DB-23 column. Compound **3A** eluted at 49.6 min and compound **3B** eluted at 50.3 min from the silica HPLC column. The KI, mass spectrum, and ^{1}H NMR data of compound **3B** were the same as that of the active compound isolated from the insect extracts. The stereochemistry of **3A** and **3B**, and the full assignment of ^{1}H and ^{13}C NMR signals, were obtained from 2D-COSY, NOESY, HMQC, and HMBC spectra as described below.

In **3A**, HMBC correlations were observed between C-1 (δ 61.1) and the proton at δ 6.29, as well as C-1 and the methine proton (δ 2.44) of the isopropyl group, indicating that the more downfield vinylic proton at δ 6.29 was H-3. In addition, the 2D NOESY NMR spectrum of **3A** (Fig. 4) indicated that the vinylic proton with a chemical shift of δ 6.08 had

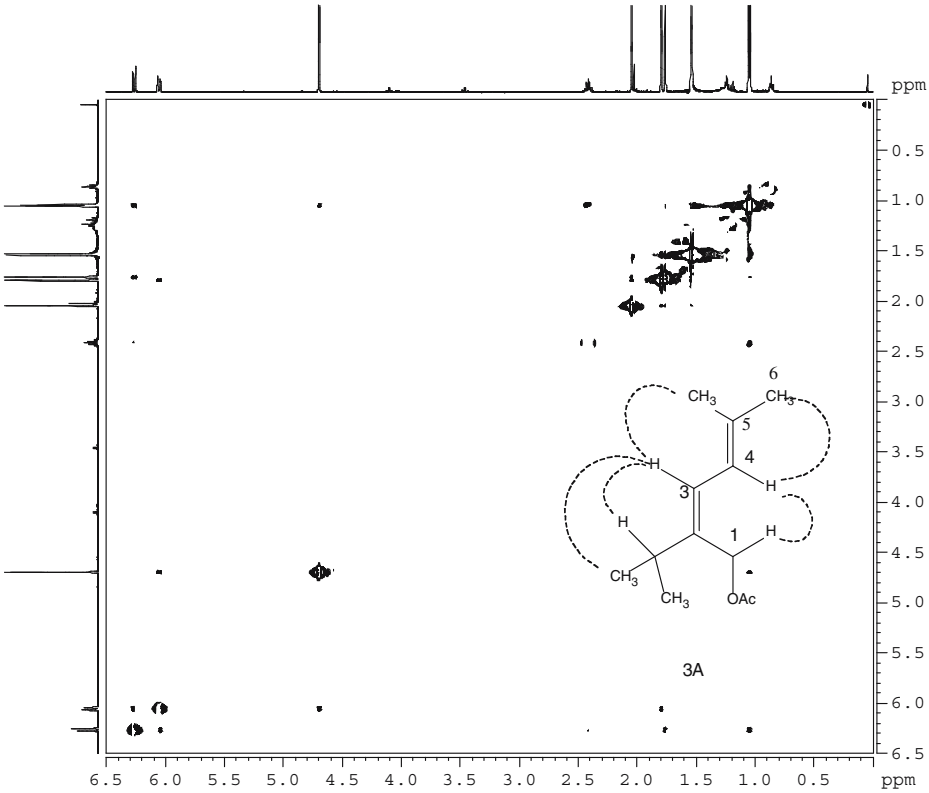


Fig. 4 2D-NOESY NMR spectrum and the key NOE correlations (shown with dashed curves) of compound **3A**

cross couplings with H-1 (δ 4.72) and H-6 (δ 1.82). The vinylic proton with a chemical shift of δ 6.29 had cross coupling with the isopropyl group (δ 1.07 and δ 2.44) and the 5-methyl group (δ 1.79). These NOE correlations suggested the configuration of the double bond in this isomer was *Z*. Thus, the structure of compound **3A** was assigned as (*Z*)-2-isopropyl-5-methyl-2,4-hexadienyl acetate. The NOE effects are summarized in Fig. 4.

Similarly, in **3B**, HMBC correlations were observed between C-1 (δ 66.6) and the proton at δ 6.23, as well as C-1 and the methine proton at δ 3.01 of the isopropyl group, suggesting that the more downfield vinylic proton at δ 6.23 was H-3. In addition, the 2D NOESY NMR spectrum of **3B** (Fig. 5) indicated that the vinylic proton with chemical shift of δ 6.08 had cross couplings with the methine proton (δ 3.01) of the isopropyl group and H-6 (δ 1.82), but not with the methyls of the isopropyl group; presumably, the two methyl groups were turned away from the vinyl proton because of steric repulsion. These data indicated that the alkenyl group (C4-C6) was *cis* to the isopropyl group. The vinylic proton with a chemical shift of δ 6.23 had cross couplings with the 5-methyl group (δ 1.79) and H-1 (δ 4.6). Thus, the downfield vinyl proton must be *cis* to the methylene proton. Thus, the structure of compound **3B** was assigned as (*E*)-2-isopropyl-5-methyl-2,4-hexadienyl acetate. The NOE effects are summarized in Fig. 5.

As a result of the matching NMR spectra, KIs on GC, and mass spectra, the pheromone of the mealybug *P. minor* was identified as (*E*)-2-isopropyl-5- methyl-2,4-hexadienyl acetate.

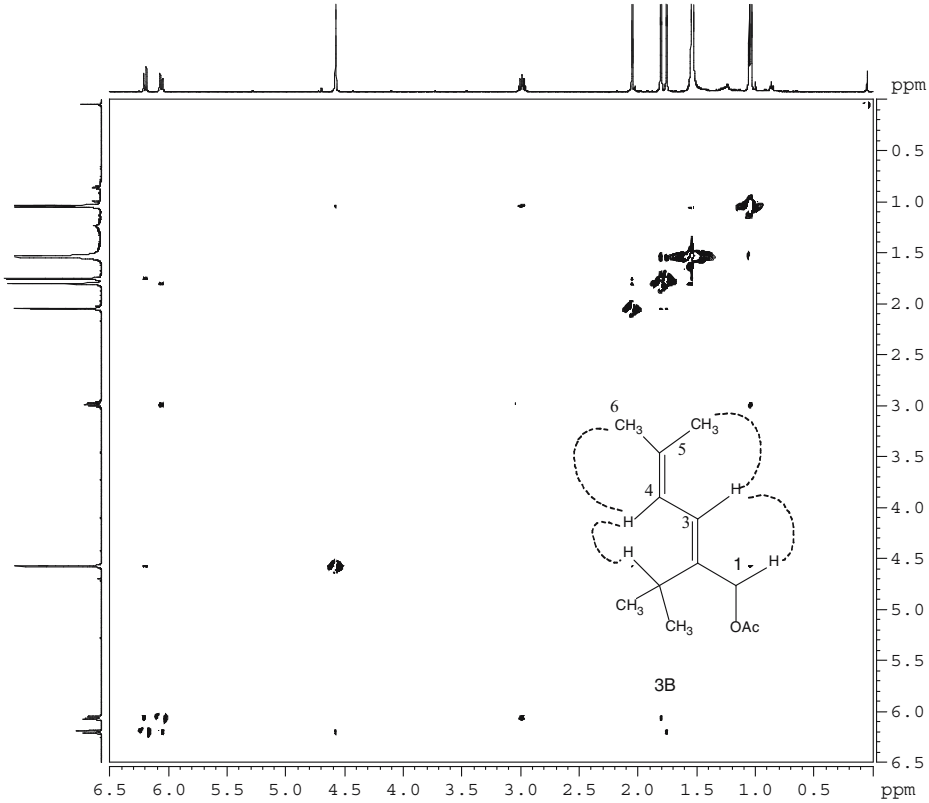


Fig. 5 2D-NOESY NMR spectrum and the key NOE correlations (shown with dashed curves) of compound **3B**

Table 1 Number of male mealybugs attracted to various treatments

Treatment	No. of Males Attracted (mean ± S. D.); N=6
Synthetic compound 3B (*E*) isomer[b] (150 ng)	91.2±19.3 a[a]
Compound isolated from mealybug volatiles (100 ng)	25.3±19.1 b
20 females on pumpkin	3.5±2.8 c
Mixture of synthetic 3A (*Z*) isomer[b] (82 ng) and 3B (*E*) isomer (58 ng)	3.2±2.6 c
Uninfested pumpkin	0 c

[a] Means followed by the same letter are not significantly different at the 5% level (Duncan's multiple range test).
[b] 2-isopropyl-5-methyl-2,4-hexadienyl acetate

Bioassay of Synthetic Pheromone The results for bioassays of the synthetic materials vs. the compound from volatiles of female mealybugs, live females, and controls are shown in Table 1. Synthetic (*E*)-2-Isopropyl-5-methyl-2,4-hexadienyl acetate was more attractive ($P<0.05$) to male mealybugs than any of the other treatments. Interestingly, the other stereoisomer appeared to antagonize attraction, with the mixture of isomers (59% of 3A [*Z*-isomer] and 41% of compound 3B [*E*-isomer]) being unattractive (i.e., not significantly different from the number attracted to the uninfested pumpkin control) to males.

Discussion

We identified the sex pheromone for the passionvine mealybug, *P. minor*, a major pest of economically important crops. Male mealybugs were attracted to the (*E*) isomer of 2-isopropyl-5-methyl-2,4-hexadienyl acetate. That male bugs were attracted in greater numbers to the pure (*E*)-isomer, rather than to a mixture of (*E*)- and (*Z*)-isomers, suggests that a stereospecific synthesis of the (*E*)-isomer will be required for practical use of this pheromone.

The structure of the sex pheromone compound of *P. minor* represents another example of the irregular terpenoid motif of the sex pheromones of pseudococcid species. The sex pheromone of *Pseudococcus viburni* (Millar et al., 2005) is a monoterpene incorporating a cyclopentane unit. The sex pheromones of *Pseudococcus cryptus* (Arai et al. 2003) and *P. citri* (Bierl-Leonhardt et al. 1981) are monoterpenes with a cyclobutane moiety. The sex pheromone of *P. ficus* (Hinkens et al. 2001) is an acyclic monoterpene with the same basic carbon skeleton as the compound we identified here. All of these species appear to use single components as attractants.

Further work will seek to develop a stereospecific synthesis of (*E*)- 2-isopropyl-5-methyl-2,4-hexadienyl acetate, as well as determine optimum doses and longevity of synthetic pheromone lures.

Acknowledgment The authors thank Penny Gullan of the University of California, Davis for preliminary identification of the species and Doug Miller and his colleagues at the US Department of Agriculture in Beltsville, Maryland, USA for confirming the species identity. The authors thank Jocelyn Millar of the University of California, Riverside for suggestions on the synthesis of the pheromones.

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Introduction

All cat species (Felidae) use their urine for communication (Macdonald 1985; Asa 1993; Mellen 1993; Ogata and Takeuchi 2000) and have two distinct patterns of behavior when depositing fluid from the urinary tract. These are *normal urination*, during which, the animal squats on the ground and releases a stream of urine, and *spray marking*, whereby urine is ejected by the animal while it stands with its hindquarters towards the target and its tail lifted vertically. The precise direction of spray varies with the species, and adult males typically spray at a higher rate than females, although adult females of most species also spray mark (Schaller 1972; Macdonald 1985; Mellen 1993; Brahmachary and Singh 2000). In both tiger, *Panthera tigris*, and lion, *P. leo*, the fluid released comprises normal urine mixed with a whitish lipid precipitate, which is also present in the bladder. The average total lipid content of the marking fluid of tiger is 1–2 mg/ml of urine (Poddar-Sarkar 1996), and as much as 20 g of lipid a day is excreted (Hewer et al. 1948). When released during normal urination, the lipids do not leave marks on the ground. When sprayed on vertical objects, however, the lipids of tiger urine stick to the surface and in time turn into a dark viscous deposit with a distinct odor (Smith et al. 1989). Odors of tiger marking fluid may last up to 3 weeks and, when fresh, can be detected up to 3 m away (Smith et al. 1989). Marking fluid from lions does not leave the same black long-lived deposit of lipids as that from tigers (Anderson and Vulpius 1999), whereas no lipids have been found in urine from cheetah, *Acinonyx jubatus*, leopard, *P. pardus*, or puma, *Felis concolor* (Asa 1993).

Intact male domestic cats, *Felis catus*, frequently spray urine that has a characteristic species-specific aroma onto vertical objects, although the locations and rates of marking vary between studies (Macdonald 1985). Felinine, a sulfur-containing amino acid (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), has been identified in the urine of several members of the Felidae including the domestic cat (Datta and Harris 1951, 1953; Westall 1953; Hendriks et al. 1995a) where urinary felinine concentration is positively correlated to blood plasma testosterone concentration. Intact males produce the greatest amount of urinary felinine (122 μmol kg^{-1} d^{-1}) compared with 41, 36, and 20 μmol kg^{-1} d^{-1} in castrated males, intact females, and castrated females, respectively (Hendriks et al. 1995b). Felinine is present in the urine from 6 mo of age (Tarttelin et al. 1998) and is detectable in the urine, kidney, and bladder (Hendriks et al. 2001). It is present in the blood as a precursor tripeptide, γ-glutamylfelinylglycine, which is a glutathione analog. Felinine production is gender-linked and is believed to be synthesized in tissues other than the kidney (Rutherfurd et al. 2002). The biological role of felinine is still unclear, but it is a urinary component involved in domestic cat spray marking. It is likely that it degrades to another compound or compounds that give rise to the distinctive "tom cat" urine odor (Hendriks et al. 1995a). Felinine has also been detected in the urine of ocelot, *F. pardalis*, Indian leopard cat, *F. bengalensis* (Datta and Harris 1951), and bobcat, *F. rufus* (Datta and Harris 1953). However, reports differ for leopards (Datta and Harris 1953; Roberts 1963), and felinine has not been found in the urine of other members of *Panthera* (Hendriks et al. 1995a; W. H. Hendriks, personal communication). Chemical signals among non-domestic cats appear to be more complex than those of the domestic cat. More than 50 different volatile compounds have been identified from lion urine, with no two individuals having exactly the same chemical composition (Anderson and Vulpius 1999). Seven compounds were found in most individual lions tested (male and female), whereas urine from males contained much more 2-butanone than that from females. Of the compounds found in only-male samples, none was detected in all males. Thus, it is unclear whether one or more of these compounds provides a male-specific signal.

Many healthy mammals exhibit an obligatory proteinuria (Monroe et al. 1989), and there is evidence that some of these proteins have a function in communication. More than 100 protein components have been identified in rat urine, including signaling proteins, enzymes, and pheromone binding proteins (Thongboonkerd et al. 2003). Cauxin (carboxylesterase-like urinary excreted protein) is a novel 70 kDa protein that is excreted in the urine of the domestic cat (Miyazaki et al. 2003), bobcat, *Lynx rufus*, and lynx, *L. lynx*, but, as yet, has not been detected in the urine of any other genus of Felidae (Miyazaki et al. 2006a). It is expressed in the epithelial cells of the straight proximal tubules and secreted from the renal tubular cells into the urine (Miyazaki et al. 2006a). Cauxin shares 40% sequence identity with a group of mammalian carboxylesterases that hydrolyze ester, thioester, and amide bonds. It is the main urinary protein of healthy male and female domestic cats, and intact males have higher levels of cauxin in their urine relative to neutered males, females, or cats of 3 mo or less (Miyazaki et al. 2003). Intact males excrete approximately 0.5 mg cauxin per milliliter of urine, decreasing by more than 90% after castration (Miyazaki et al. 2006a). Recently, Miyazaki et al. (2006b) provided evidence for the activity of this protein as a peptide hydrolase, acting on the dipeptide felinylglycine and releasing felinine into the urine. This raises the possibility that cauxin is an important component of sexual signaling in domestic cats and possibly other Felidae, functioning to control the expression of felinine in urine scents. In this study, we report the further characterization of cauxin from both domestic cats and a number of species of big cats. In marked contrast to previous studies (Miyazaki et al. 2006a), we provide proteomic-based evidence that cauxin is expressed by *Panthera* species, which are not thought to produce felinine or its precursors. This suggests that the esterase/peptidase might have other roles in the elaboration of urinary scents in this group of cats.

Methods and Materials

Urine samples from 13 mature intact domestic cats (five male and eight female; aged from 6 mo to 15 yr) were collected by cystocentesis, free catch, or by free collection from the floor. Urine samples from one individual of each of five species of mature male non-domestic cats (Asiatic lion, *Panthera leo persica*; Sumatran tiger, *P. tigris sumatrae*; Persian leopard, *P. pardus saxicolor*; jaguar, *P. onca*; and clouded leopard, *Neophilis nebulosa*) and one mature Asiatic lioness were collected by catheterization during a routine health check at Chessington World of Adventure, Surrey, UK. Samples were centrifuged to remove cell debris and stored at −20°C until required.

Urine protein content was measured using the Coomassie plus protein assay (Perbio Science UK Ltd., Tattenhall, UK). Creatinine levels were also determined to correct for urine dilution using a modification of the Jaffe method of analysis (Cook 1971). Before gel electrophoresis, samples from domestic cats were concentrated, and low molecular weight compounds were removed by acetone precipitation. To each sample, a fivefold volume of acetone was added and incubated for 1 hr at −20°C, centrifuged, 5,000×*g* for 5 min, the supernatant removed, and the pellet resuspended. Urine samples from big cats were treated with StrataClean™ Resin (Stratagene®, Hycor Biomedical Ltd., Edinburgh, UK), which binds and concentrates the protein in the sample and removes non-protein impurities. The resin was added to each sample, vortexed for 1 min then centrifuged at 5,000×*g* for 5 min. The supernatant was removed, the pellet washed with distilled, deionized, water and resuspended.

One-Dimensional Gel Electrophoresis Samples (10 μg) of the urine were electrophoresed through a 12% polyacrylamide gel according to previously documented methods (Laemmli 1970). A 12% resolving gel was prepared and allowed to polymerize before overlaying with a 4% stacking gel. Samples were incubated for 30 min at 37°C in either a reducing (Tris–HCl, 0.125 M; SDS, 0.14 M; glycerol 20% *v*/*v*; dithiothreitol, 0.2 mM and bromophenol blue, 0.03 mM) or non-reducing buffer before loading. The proteins were electrophoresed at a constant potential of 200 V until the dye front had progressed to the bottom of the gel. Gels were stained with Coomassie blue (Bio-Safe™, BioRad, CA, USA).

Two-Dimensional Gel Electrophoresis Before loading onto IPG strips, pH 3-10L (GE Healthcare UK Ltd., Little Chalfont, UK,) each sample (300 μg protein) was treated with StrataClean™ Resin. The resulting pellet was resuspended in buffer containing CHAPS (4% *w*/*v*), 7 M urea, 2 M thiourea, 20 mM dithiothreitol, and ampholytes (0.5% *v*/*v*) and incubated at room temperature for 1 hr. After centrifugation, the supernatant was separated in the first dimension on an IPGPhor unit (AP Biotech Pharmacia). In-gel rehydration (150 Vh at 30 V, 300 Vh at 60 V, 20°C) was followed by isoelectric focusing (500 Vh at 500 V, 1,000 Vh at 1,000 V, and 48,000 Vh at 8,000 V). The focused IPG strips were equilibrated in 50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% (*v*/*v*) glycerol, 2% (*w*/*v*) SDS, and a trace of bromophenol blue. Dithiothreitol (10 mg/ml) was present as a reducing agent for the initial equilibration, and iodoacetamide (25 mg/ml) was present in the second equilibration step. The proteins were electrophoresed through a linear 12.5% acrylamide gel, and the gels were stained using Coomassie blue (Bio-Safe™, BioRad).

In-Gel Trypsin Digest Gel plugs containing protein from areas of interest were excised, and the proteins were subjected to in-gel tryptic digestion and peptide extraction using a MassPrep™ digestion robot (Waters, Manchester, UK). Briefly, excised gel plugs were placed in distilled deionized water (50 μl) before addition of ammonium bicarbonate (50 μl, 100 mM) and acetonitrile (50 μl). This liquid was removed, and the gel piece was treated with destain solution [10 μl of 50% (*v*/*v*) acetonitrile, 50% (*v*/*v*) 100 mM ammonium bicarbonate]. The protocol included a second cycle of reduction and alkylation. The gel plug was dehydrated in acetonitrile and then rehydrated with 25 μl of trypsin (3 ng/μl) in buffer. After digestion (5 hr), tryptic peptides were extracted from the gel matrix by addition of 30 μl of 2% (*v*/*v*) acetonitrile/1% (*v*/*v*) formic acid. Peptides (1 μl) were mixed with matrix (1 μl) [α-cyano-4-hydroxycinnamic acid saturated solution in a 1:1:1:1 by volume of ethanol, acetonitrile, trifluoroacetic acid (0.4%), and distilled deionized water], and the resultant 2 μl was spotted onto the MALDI target. ACTH (adrenocorticotropic hormone, M=2,464.20), 1 μl of 1 μg/ml stock was used as an external lockmass standard.

MALDI-ToF Mass Spectrometry Peptides were analyzed by using a matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF MS) mass spectrometer (M@LDI™, Waters) over the *m/z* range of 1,000 to 3,500 thompsons. The mass spectrometer was calibrated with 2.5 pmol each of bradykinin (MW=903.47), neurotensin (MW=1,671.92), ACTH (MW=2,464.20), and insulin β-chain (MW=3,493.65). Typically, a residual error of ±0.05 ppm was obtained. The mass accuracy of spectra could be further improved by internal standardization with the major trypsin autolysis peak ($[M+H]^+$=2163.057 thompsons). Proteins were identified from their peptide mass fingerprint by manual searching using a locally implemented MASCOT server (ver 1.9, http://www.matrixscience.co.uk) against the Swiss-Prot http://expasy.org/sprot/) or MSDB http://csc-fserve.hh.med.ic.ac.uk/msdb.html/) databases. The initial search parameters

allowed a single missed tryptic cleavage, obligatory (fixed) carbamidomethyl modification of cysteine residues, variable oxidation of methionine, and an *m/z* error of ±250 ppm. The taxonomic search space was restricted to Mammalia. Before MALDI-ToF MS analysis, residual contaminants were removed if necessary from exotic cat samples using ZipTip® C18 tips (Millipore, Hertfordshire, UK).

Electrospray Ionization Mass Spectrometry Electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) were performed on a Q-ToF Micro instrument (Waters-Micromass) fitted with a nanospray source. The electrospray was created from a silver-coated glass capillary with a 10-μm orifice (Presearch, Hitchin, UK) held at a potential of +2,000 V relative to the sampler cone. Before ESI-MS/MS, peptides were separated by reversed phase high-performance liquid chromatography on a Dionex Ultimate system. The unit was fitted with a PepMap C18 column (LC packings), 15 cm×75 μm, bead size 3 μm and pore size 100 Å. Before separation, aliquots (10–20 μl) of sample digest were taken up into the injection loop of the Dionex Famos autosampler and desalted in-line using a Dionex Switchos apparatus fitted with a 5 mm× 300 μm C18 pre-column. The pre-column was initially equilibrated in 0.2% (*v*/*v*) formic acid at 30 μl/min. Peptides were then loaded and washed for 3 min at the same rate, after which, the trap and downstream PepMap column were developed with 90% acetonitrile/0.2% formic acid, introduced as a linear gradient of 0–50% in 30 min at 0.2 μl/min. The column eluant was monitored for UV absorbance at 214 nm in an in-line flow cell before being introduced into the mass spectrometer. The initial quadrapole analyzer was set to allow the transmission of selected precursors into the gas cell. The masses of the resulting fragment ions were then measured by the ToF analyzer. Precursor spectra were acquired between *m/z* 400 and 1,500 at a scan/interscan speed of 2.4/0.1 sec. Product ion spectra were acquired between m/m 100 and 2,000 at a scan speed of 1.0/0.1 sec. Raw product ion spectra were deconvoluted using the MaxENT algorithm within the Mass Lynx software. Interpretation of product ion spectra and the determination of peptide sequences were facilitated by the PepSeq module within Mass Lynx. Peptide sequences were searched using the National Center for Biotechnology Information basic local alignment search tool (BLAST). The search was limited to Mammalia; no other parameters were limited.

Results and Discussion

Urine samples from a range of domestic cats each contained multiple proteins distributed between 5 and 300 kDa (Fig. 1a). The overall pattern of bands from one-dimensional gel electrophoresis was similar among individuals, and there was no pronounced difference between males and females. Prominent bands were evident at a mobility corresponding to a molecular weight of approximately 66 kDa, but other bands were also present. To determine the identity of these protein bands, small plugs of gel (1 mm^2) corresponding to each band were recovered, instilled with trypsin, and the resultant tryptic peptides were recovered and analyzed by MALDI-ToF mass spectrometry (peptide mass fingerprinting, results not shown) and electrospray ionization mass spectrometry. This permitted the identification of several of the bands. Predominant urinary proteins included uromodulin (Tamm-Horsfall glycoprotein) present as a diffuse band at 120 kDa and transferrin at approximately 75 kDa. The most intense band in almost every sample migrated at approximately 67 kDa, and might have been anticipated to be serum albumin—urinary

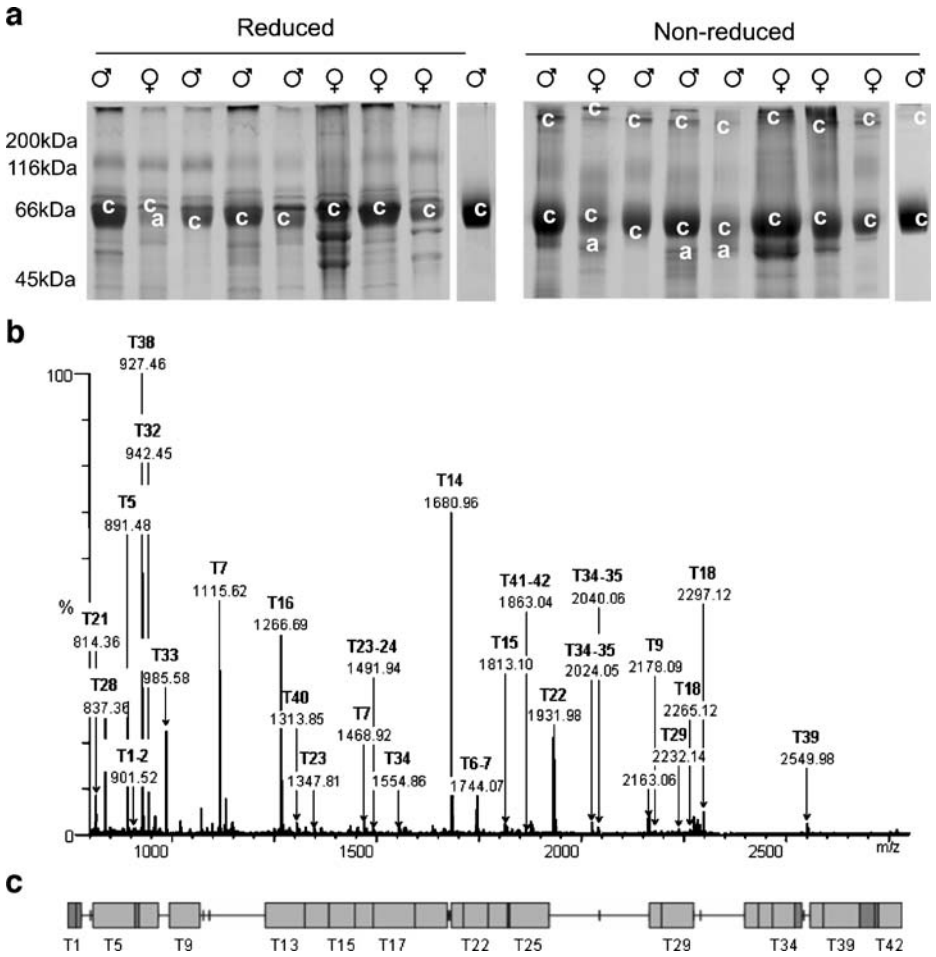


Fig. 1 Analysis and identification of urinary proteins from the domestic cat, *Felis catus*. Proteins in urine samples from adult male and female domestic short hair cats were resolved by reducing and non-reducing one-dimensional SDS-PAGE (**a**). Protein bands in the highly stained region of 60 kDa were excised and subjected to gel digestion before MALDI-ToF mass spectrometry. The proteins identified as cauxin (*white c*) and albumin (*white a*) are indicated on the gel. The mass spectrum of the band identified as cauxin is presented (**b**), and each tryptic peptide from the MALDI-ToF spectrum is highlighted in the peptide map (**c**)

serum albumin is a common observation in renal dysfunction. However, peptide mass fingerprinting revealed a complex series of tryptic peptides that matched with a very high score to the protein cauxin (Entrez Protein ref. BAC22577). Some peptides were also consistent with the presence of feline serum albumin, but it is likely that most of the protein in this band is cauxin. Cauxin is a single polypeptide chain 67 kDa, but exists in part as disulfide-linked multimers. When urine samples were resolved in non-reduced gels, a new series of bands migrating from 300 to 350 kDa was resolved. MALDI-ToF analysis of these bands confirmed that each of them contained tryptic peptides from cauxin. In contrast, albumin, which does not exist as disulfide-linked multimers, remained at a position on the gel approximating 60 kDa. The multiple high-mass cauxin bands might reflect N-linked glycosylation, as the sequence contains four potential glycosylation motifs

(Asn-X-Ser/Thr). Alternatively, the different bands could reflect disulfide-linked association between differentially proteolyzed subunits. Further evidence for cauxin being the predominant protein at the 66 kDa region of the gel was derived from two-dimensional gel electrophoresis (results not shown). After separation, a major protein staining area, smeared over the pI region from 3.5 to 5.8, was identified as cauxin. A smaller, more intense discrete spot, at pI 4.7, was identified by peptide mass fingerprinting as serum albumin.

The tryptic peptide fragments were subjected to *de novo* peptide sequencing (Fig. 2). Peptides were resolved by C18 reversed phase capillary chromatography, and specific peptides were fragmented by collision-induced decomposition. The product ion spectra were then used to direct manual peptide sequencing. Thirteen peptides were sequenced this way. These peptide sequences provide unambiguous confirmation that the protein is cauxin. One tryptic peptide in particular was readily and repeatedly sequenced ($[M+H]^+$=1,115.6 *m/z*) as PALPWNDFR, a sequence that explains all of the mass of the peptide (predicted $[M+H]^+$=1,115.56). This sequence was significant for two reasons. First, the equivalent tryptic peptide expected from cDNA sequencing was *QPK*PALP*G*NDFR (Miyazaki et al. 2003). The glycine residue (G) in this cDNA sequence is at variance with other reported cauxin-like sequences from several species, from which, the consensus *P*x*P*xxx*W* is evident (Ecroyd et al. 2006), and the peptide sequence obtained here corrects a previously reported anomaly possibly because of a cDNA sequencing error. The DNA sequence used to derived the previously published cat sequence was CCC[GGG]AATGACTTCCGA; for tryptophan (W) to be coded for in the same sequence, the DNA sequence would have to be CCC [GGA]AATGACTTCCGA, a single base shift near a region of potential GC compression. The second significant feature of this peptide is that it has nominally been generated by tryptic proteolysis, from the primary sequence context—KQPK[*palPWNDFR*]NAT—trypsin is not generally considered to be capable of cleaving peptide bonds in which the amino acid C-terminal to the scissile bond is proline (Olsen et al. 2004). Thus, the most likely explanation is that cauxin is trimmed by additional N-terminal processing possibly related to the secretion into urine. The proximal tubule of the kidney is replete with exo- and

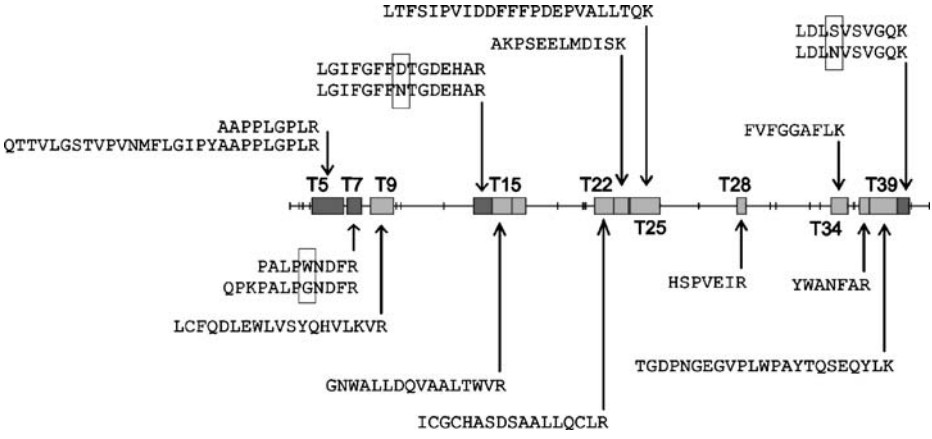


Fig. 2 *De novo* peptide sequencing of tryptic peptides of cauxin from the domestic cat, *F. catus*. Tryptic peptides from an in-gel digest of a band identified by peptide mass fingerprinting as cauxin were resolved using nanoflow reversed phase chromatography, fragmented by tandem mass spectrometry, and the product ion spectra were interpreted manually to obtain the peptide sequence data. The peptides for which sequence data were obtained are highlighted on the peptide map, and peptides that differed from the published sequence are highlighted (this study, top line, previously published sequence, accession number BAC22577)

endopeptidases that could be responsible for this processing. This processing must be partial, however, as we also found clear sequence data for an additional peptide that was derived from cauxin and that was N-terminal to the sequence PALPWNDFR. The multiple bands on non-reducing gels are also indicative of mass heterogeneity in cauxin subunits.

Cauxin has the signature motifs and overall sequence that places it in the class of carboxylesterases (Miyazaki et al. 2003). Although the function of cauxin is not known, it has been suggested that it is responsible for the elaboration of the strong scent of feline in urine used in territorial marking (Miyazaki et al. 2006b). Urinary scent marking is a feature of many members of the Felidae, and accordingly, we examined urine samples from a number of species of big cats for the presence of cauxin, including Asiatic lion, Sumatran tiger, jaguar, Persian leopard, and clouded leopard. In all instances, urine samples were obtained from intact adult males. In all of the male cats, a band corresponding in mass to cauxin was evident, although in several of the samples, the presence of urinary serum albumin migrating at approximately the same position on the gel made identification of cauxin more difficult (Fig. 3). However, when the gels were run under non-reducing conditions, high-mass complexes were evident (Fig. 3a), and these yielded tryptic peptides that could be sequenced readily by tandem mass spectrometry. Multiple peptides were analyzable, which, interestingly, were the peptides cognate to those seen in the domestic

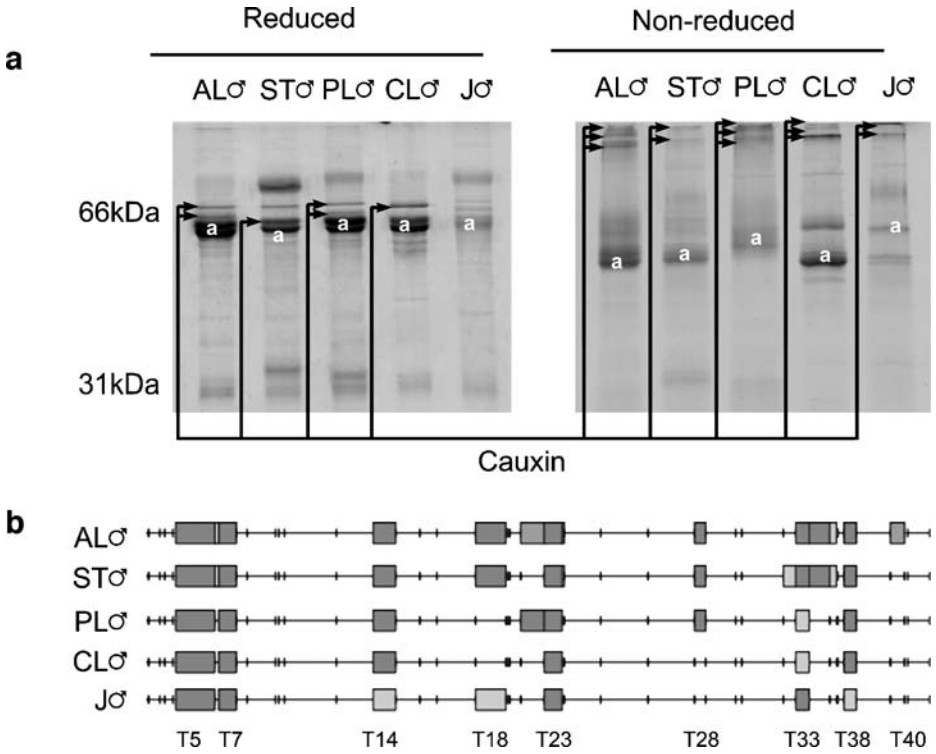


Fig. 3 Analysis and identification of male urinary proteins from the Asiatic lion (*AL*), Sumatran tiger (*ST*), Persian leopard (*PL*), clouded leopard (*CL*), and jaguar (*J*). Proteins in urine samples were resolved by reducing and non-reducing one-dimensional SDS-PAGE (**a**). The proteins identified as cauxin and albumin (*white a*) are indicated on the gel. Major protein bands were excised and subjected to in-gel digestion before peptide mass fingerprinting and the peptides observed by mass spectrometry are highlighted (**b**)

cat. This implies that these were similar peptides that yielded strong ion currents and good fragmentation data irrespective of the source (Fig. 4). Although multiple peptides were sequenced for each of the big cats, there were remarkably few amino acid changes within the big cats, or indeed, when compared to the domestic cat, from which, we infer a high degree of conservation of structure, activity, and function for this protein (Fig. 5). Equally intriguing, the unusual tryptic peptide PALPWNDFR, previously discussed, was present in all big cat samples, implying a similar pattern of N-terminal processing or unusual tryptic cleavage in different species (Fig. 5). The sequence of cauxin from each of the big cats is noticeably similar to that of the domestic cat. We compared this degree of sequence similarity with that obtained for serum albumin also present in urine samples (Fig. 6). For

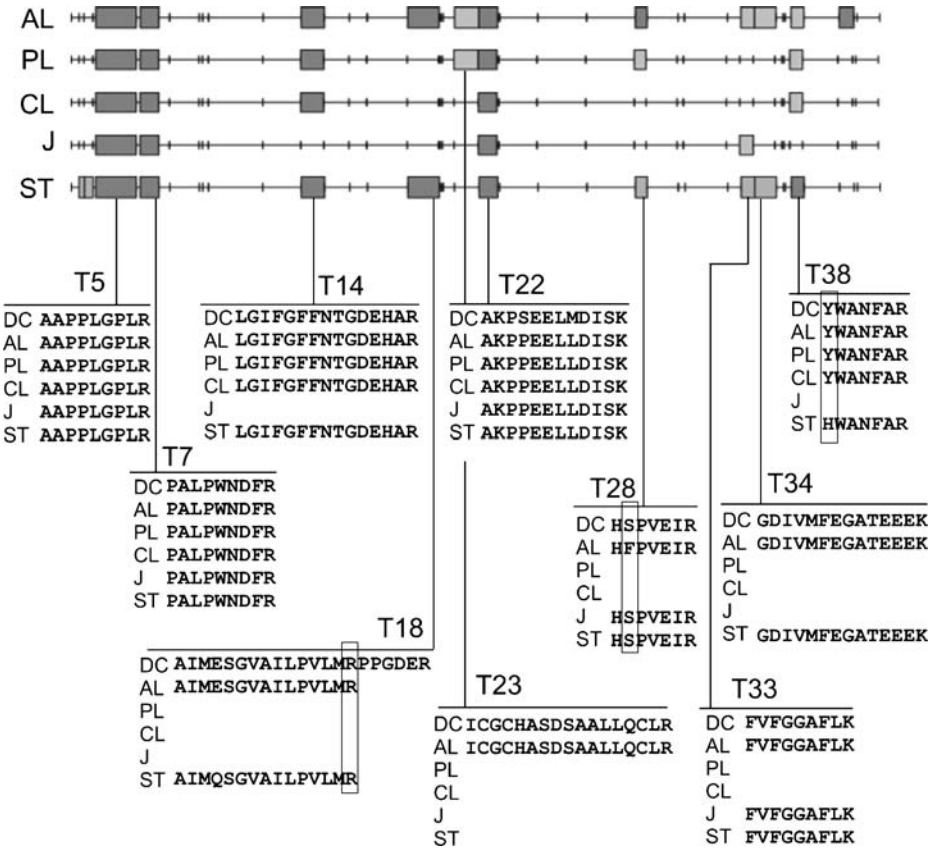


Fig. 4 De novo peptide sequencing of tryptic peptides of cauxin from big cats. Urine samples from five big cat species were resolved on SDS-PAGE (abbreviations as Fig. 3; *DC* domestic cat). Tryptic peptides from an in-gel digest of the band identified by peptide mass fingerprinting as cauxin were resolved using nanoflow reversed phase chromatography, fragmented by tandem mass spectrometry, and the product ion spectra were manually interpreted to obtained peptide sequence data. The peptides for which sequence data were obtained are highlighted on the peptide map. Most peptides had the same sequence as those for the domestic cat, but where peptide sequences differed from the domestic cat in at least one of the big cat species, the amino acid differences are *boxed*. For tryptic peptide T18 from the Asiatic lion (*AL*) and the Sumatran tiger (*ST*), the termination of the sequence at the arginine residue, which in domestic cat is extended to include a non cleavable ArgPro sequence, is presumptive evidence for an amino acid change at that position

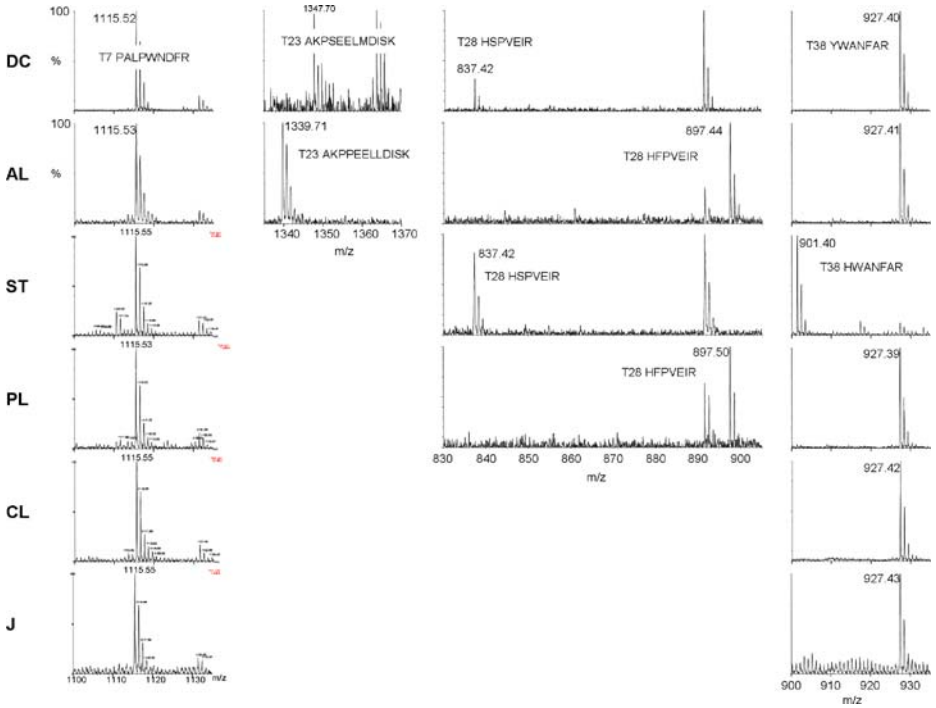


Fig. 5 Identification of polymorphic peptides in cauxin species for big cat species. Urine samples from five big cat species were resolved on SDS-PAGE (abbreviations as Fig. 3, *DC* domestic cat). Tryptic peptides from an in-gel digest were sequenced by tandem mass spectrometry, and for selected peptides where different sequences were obtained for big cats, the corresponding $[M+H]^+$ ion from MALDI-ToF mass spectrometry is shown, together with the corresponding ion that confirms the amino acid change. Additionally, the atypical tryptic fragment PALPWNDFR was observed in all six cat species, and the MALDI-ToF mass spectrum for that specific $[M+H]^+$ ion is included in the *left hand* column

domestic cat, a total of 50 tryptic peptides were identified, covering approximately 66% of the sequence. For the Asiatic lion, 34 tryptic peptides derived from albumin were identical to the domestic cat (covering 58% of the sequence); the corresponding figures for the clouded leopard and the jaguar were 38 (63% coverage) and 36 (51% coverage), respectively. In both the Sumatran tiger and the Persian leopard, we identified 43 (64% coverage) and 39 (59% coverage) tryptic peptides, respectively, but for two peptides (Sumatran tiger) and three peptides (Persian leopard), single amino acid changes were observed. The degree of sequence conversation in serum albumin is high and reflects the relatedness of these species. It is, therefore, not surprising that cauxin has a similar degree of conservation, but this also implies that mechanisms of sexual selection are not driving species-specific evolution of this protein.

The expression of cauxin by several *Panthera* spp. was unexpected, as a previous study had reported that cauxin was not present in urine samples (Miyazaki et al. 2006a). By contrast with the intact domestic male cat (Fig. 1, and Miyazaki et al. 2003); the band intensity of cauxin in these big cats was substantially lower. The protein-based evidence for the existence of cauxin as a urinary component is substantial: identification based on peptide mass fingerprinting, which requires a high degree of sequence conservation among the species under investigation and a model species for which a database entry already

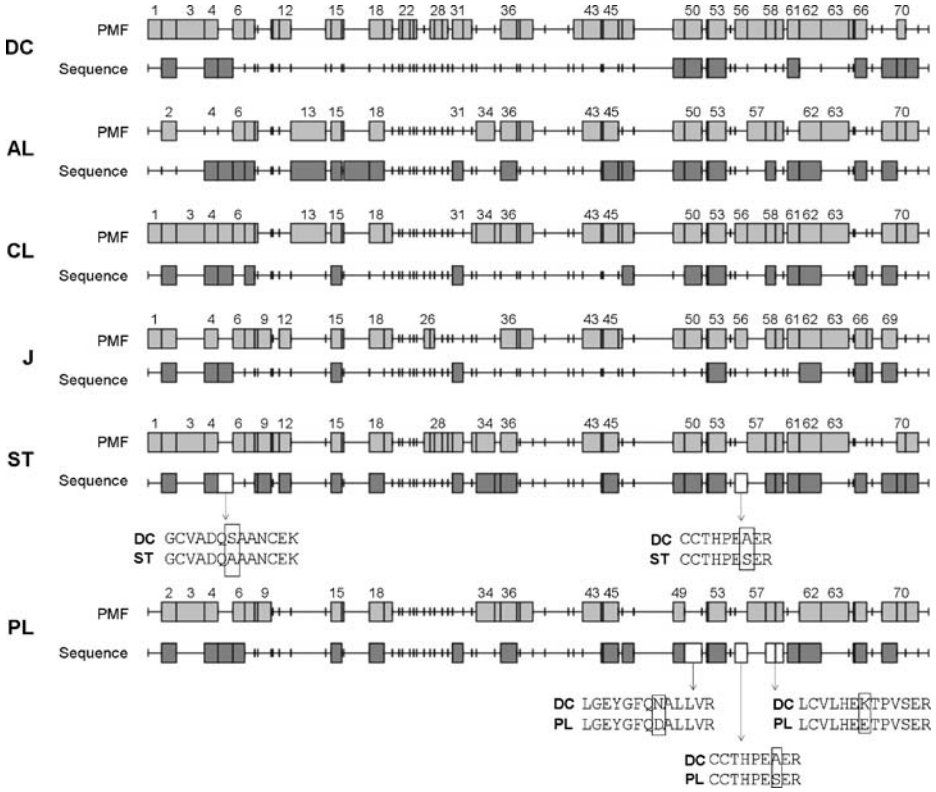


Fig. 6 Sequence homology of albumin in domestic and big cat species. Urine samples from five big cat species were resolved on SDS-PAGE. Tryptic peptides from an in-gel digest of a band putatively identified as serum albumin were used to confirm the identity of this protein. Peptides matched by mass alone (peptide mass fingerprinting) are highlighted as *boxes* in the PMF row for each species (abbreviations as Fig. 3). Additionally, selected peptides were resolved by nanoflow reversed phase chromatography, fragmented by tandem mass spectrometry, and the product ion spectra were manually interpreted to obtained peptide sequence data (row marked 'Sequence'). Where peptide sequences were identical to those for the domestic cat, no additional information is provided, but where sequence differences were observed, the changed sequence and the corresponding sequence for albumin from the domestic cat are *aligned*, with differences *boxed*

exists, and *de novo* peptide sequencing, which can be used as a probe in BLAST searches. In contrast, the previous investigation was based on Western blotting, with an antibody to a C-terminal peptide of cauxin in the domestic cat (SVGQKLKEQEVEFWMNTIVP, spanning tryptic peptides T40 to T42). It is possible that this peptide might not be so highly conserved in the big cat species, and thus, Western blotting might yield weaker signals, which would be exacerbated by the much lower level of expression of cauxin in the big cats.

It is likely that the role of cauxin in the domestic cat is not to act as a carboxylesterase, but as an amidase that catalyzes the hydrolysis of the dipeptide 3-methybutanol-cysteinylglycine to glycine and the cat-specific amino acid felinine. Felinine and its degradation products are putative pheromones (Miyazaki et al. 2007), and the role of cauxin in elaboration of the pheromone from a precursor peptide is enigmatic. What then of the expression of the homologous enzyme in big cats? Neither felinine nor its precursors are present in urine of the Pantherinae (Hendriks et al. 1995a; W.H. Hendriks, personal

communication), and the role of cauxin in these species, albeit in reduced amounts, remains enigmatic. It is possible that the cauxin homolog acts as an amidase, but with different precursor molecules. However, some cats express urine that is replete with complex fatty acyl esters, and alternative substrates should be considered. It is clear that the pheromone/enzymology axis of cats is at the beginning of a new and fascinating phase of development.

Acknowledgments This work was supported by a grant from Pet Plan Charitable Trust.

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Introduction

Chemical defenses are widely distributed in insects and play a crucial role for their protection against attack by predators (Spencer 1988; Trigo 2000; Ruxton et al. 2004). They can act in a direct way on the predator by inflicting pain or poisoning, or indirectly by association with aposematism (Ruxton et al. 2004). Although many of the defensive chemicals of phytophagous insects are toxins sequestered directly from the host plant on which the larva or adult feeds (Duffey 1980; Rowell-Rahier et al. 1991; Nishida 2002), insects can also synthesize *de novo* their own toxins (Pasteels et al. 1992; Mardulyn et al. 1997; Feld et al. 2001; Hartmann et al. 2004; Soe et al. 2004). We are beginning to understand the importance of diversity in defense chemistry. For instance, differences in chemistry between mimetic species may promote learning by predators (Skelhorn and Rowe 2005). Variation in the levels of defense within a species (resulting in the phenomenon of automimicry) is often associated with costly forms of defense (Bowers 1992; Fordyce et al. 2006). Much less is known about the sources of intraspecific variation in the composition of defense chemistry. For sequestered compounds, there is evidence for geographical variation in both the concentration and composition of defenses, often associated with shifts in host-plant use (Kopf et al. 1998; Moranz and Brower 1998; Wahlberg 2001). However, when the defense chemicals are synthesized by the insects themselves, the level of variation and its ultimate explanation still remain unknown. In this study, we examined the extent to which (1) environmental conditions and (2) genetic inheritance modify the composition of the defense secretion in an alpine leaf beetle.

The phytophagous genus *Oreina* Chevrolat (Coleoptera: Chrysomelidae) includes 25 species found throughout the mountain regions of Europe with some incursions into the lowlands and into western Siberia. Most are oligophagous on either Apiaceae or Asteraceae. Four species feed on both families and have been considered as polyphagous, whereas monophagy seems scarce but does exist (*O. gloriosa* on *Peucedanum ostruthium*, for instance) (Jolivet et al. 1986).

Chemical defense is a widespread strategy in Chrysomelidae and is found in all *Oreina* species (with the exception of *O. melanocephala*; Dobler et al. 1996). Adults use active secretion with exocrine glands situated on the pronotum and the elytra, whereas larvae (which lack exocrine defensive glands) stock chemicals in their body (Dobler and Rowell-Rahier 1994). All species that feed on Apiaceae presumably synthesize cardenolides autogenously from ubiquitous plant sterols as shown in closely related chrysomelids (Pasteels and Daloze 1977; Van Oycke et al. 1987). A few *Oreina* species that feed on Asteraceae also sequester pyrrolizidine alkaloid (PA) N-oxides from their host plants, whereas one species (*O. cacaliae*) has lost the ability to produce cardenolides (Dobler et al. 1996). The production of cardenolides seems to be the ancestral character shared with many other species of Chrysomelinae, with PA sequestration having evolved in just a few species of *Oreina*.

In this study, we focused on *Oreina speciosa*, which feeds on several genera of Apiaceae and produces only autogenous cardenolides. We aimed to determine the extent of geographical variation in defense chemistry and whether variation in the cardenolides secreted by *O. speciosa* is related to environmental conditions and/or is genetically inherited. The species can be found throughout Europe in the Alps, the Jura, the French Massif Central, the Balkans, and the Vosges. With at least 38 different components in its secretion (Rowell-Rahier and Pasteels 1994), *O. speciosa* possesses the most complex blend in the genus *Oreina*. Moreover, a comparison of two populations showed differences in their cardenolide profiles (Rowell-Rahier and Pasteels 1994).

A few studies—focused on quantitative genetics—have measured genetic and phenotypic variation of chemical defense in beetles (Eggenberger and Rowell-Rahier 1992; Holloway et al. 1993). Although a considerable part of the variation seems to be genetically determined (e.g., the average heritability of the concentration of 16 secretion compounds in *O. gloriosa* was estimated to be 0.45; Eggenberger and Rowell-Rahier 1992), environmental differences among locations (climate, food quality, predation pressure) are also likely to contribute to the diversity. However, to our knowledge, no studies have tested for a relationship between genetic divergence, environmental conditions, and the composition of chemical defenses.

Therefore, we aimed to determine whether the composition of defense secretions is genetically inherited or shaped by the environment. We analyzed the blend of cardenolides in populations of *O. speciosa* across Europe and correlated this variation with the genetic structure among populations and environmental differences among sites.

Methods and Materials

Collection of beetles and ecological data Sampling of *O. speciosa* was carried out in 2005 at 18 sites in France, Italy, Switzerland, Slovenia, and Croatia (Table 1), covering the Balkans (late June), the Jura (late July), the French Massif Central (mid August), and the Alps (throughout the collecting period, until late August). The latitude, longitude, and altitude of all sites were registered with a global positioning system receiver. The type of habitat was described in each site according to (1) the kind of adjacent vegetation (megaphorbiae or grasslands), (2) the dominant tree species in the surrounding forest (larch [*Larix decidua*], beech [*Fagus sylvatica*] or fir [*Abies alba*]), and (3) the presence/absence of a stream nearby. The host plants on which the beetles were collected were also recorded. Based on these observations, two distance matrices were constructed by using the "R Package for Multivariate and Spatial Analysis" version 4 (Casgrain and Legendre 2001). A habitat distance matrix was created by computing the Jaccard indexes among sites based on the three factors described above (Jaccard 1900). A host-plant distance matrix was derived from the Jaccard indexes based on host-plant sharing.

After collecting their defensive secretions (see below), the beetles were determined by examination of morphological traits under a binocular microscope (Lohse and Lucht 1994). They were then put separately in 1.5-ml labeled Eppendorf tubes containing pure ethanol and stored at −20°C.

Collection of defense secretions Defensive secretions of freshly field-collected beetles were collected from each population. To get enough liquid for analysis, secretions from 5 to 10 insects were pooled. Secretions were obtained by holding the beetles under a binocular microscope and gently tapping on the elytra with forceps until they secreted drops from both sides of the prothorax. Drops were collected with a glass microcapillary and immediately placed in a tube containing approximately 1 ml of methanol. The tubes were stored at −70°C to avoid degradation.

Chemical preparation of samples and chromatographic analysis After crushing the microcapillary with a micropestle, samples were centrifuged for 10 s at 9,000 rpm (5,970×*g*) to eliminate glass fragments, and the liquid was transferred into new tubes. The solutions were concentrated under a nitrogen flow to a final volume of approximately 200 μl. Sample analyses were carried out with the reverse phase-high performance liquid

Table 1 Collection sites of *O. speciosa* with their environmental characteristics

Site	Code	Coordinates	Altitude	No. Carden[a]	Vegetation	Habitat Trees	Stream	Host Plants
Crêt de la Neige, F	FR1	46°16′05.0″N 05°57′00.0″E	1715	38	M	–	–	Hsp, Lla
Puy Mary, F	FR8	45°06′40.9″N 02°40′51.8″E	1550	36	M	–	+	Ags, Pos
Puy de Dôme, F	FR9	45°46′07.7″N 02°57′33.7″E	1292	19	G	–	–	Ags, Sli
Ailefroide, F	FR13	44°53′41.5″N 06°26′44.7″E	1600	42	M	*Larix*	+	Asy, Pos
Abriès, F	FR14	44°48′49.1″N 06°58′28.3″E	1886	32	M, G	*Larix*	–	Asy, Cvi, Hsp, Pos
Risnjak, HR	HR2	45°25′39.5″N 14°37′19.4″E	1402	27	M, G	*Abies, Fagus*	–	Hsp, Lla
Velebit, HR	HR4	44°48′28.0″N 14°58′14.7″E	1422	34	G	–	–	Hsp
Col du Pt St-Bernard, I	IT1	45°42′12.0″N 06°52′29.4″E	1996	38	M	–	+	Pos
Predmeja, SLO	Slo2	45°55′56.7″N 13°50′31.2″E	1142	33	M	*Abies, Fagus*	–	Hsp
Bohinjsko jezero, SLO	Slo9	46°16′58.1″N 13°47′13.4″E	1261	30	M	*Fagus*	–	Asy, Hsp
Vršič pass, SLO	Slo10	46°25′29.0″N 13°44′34.6″E	1387	26	M	*Abies, Fagus*	+	Asy, Hsp
Lac Lioson, CH	VD1	46°23′13.9″N 07°07′48.6″E	1843	37	M	*Abies*	+	Asy, Hsp, Pos
Sanetsch, CH	VS2	46°18′04.8″N 07°19′47.8″E	1680	37	M	*Larix*	–	Asy, Cvi, Hsp
Saas Almagell, CH	VS4	46°04′42.3″N 07°57′32.0″E	1620	26	M, G	*Larix*	+	Ags, Asy, Cvi, Hsp, Pos
Les Haudères, CH	VS5	46°04′52.0″N 07°30′18.1″E	1436	27	M	–	+	Asy, Hsp, Pos
Emosson, CH	VS6	46°03′55.3″N 06°55′43.9″E	1944	30	M	–	–	Hsp, Pos
Chandolin, CH	VS7	46°14′41.2″N 07°36′09.3″E	2000	31	M	*Larix*	–	Pos
La Fouly, CH	VS8	45°56′10.2″N 07°05′36.1″E	1571	36	M	*Abies*	–	Asy, Pos

F: France, *HR*: Croatia, *I*: Italy, *SLO*: Slovenia, *CH*: Switzerland, *M*: Megaphorbiae adjacent vegetation, *G*: grasslands adjacent vegetation, *Ags*: *Angelica sylvestris*, *Asy*: *Anthriscus sylvestris*, *Cvi*: *Chaerophyllum villarsii*, *Hsp*: *Heracleum sphondylium*, *Lla*: *Laserpitium latifolium*, *Pos*: *Peucedanum ostruthium*, *Sli*: *Seseli libanotis*, +: stream present, –: stream or tree absent

[a] Number of cardenolides detected.

chromatography (RP-HPLC) method coupled to mass spectrometry (MS). Characteristics of both devices and analysis conditions are described below. (1) RP-HPLC: Agilent 1100 series LC (Agilent Technologies, Basel, Switzerland); G1379A degasser; G1312A binary pumps; G1313A autosampler; G1316A column compartment; G1315A diode array detector; reverse phase C18 column (Lichrospher® 250×4 mm, 5 μm, 100 A [MERCK, Darmstadt, Germany]); reverse phase-18 guard column Lichrocart® 4-4 [MERCK,

Darmstadt, Germany]; flow rate of 0.45 ml/min; solvent system of deionized water and acetonitrile (CAN, HPLC grade) gradient over 60 min (with acetonitrile increasing from 15% to 42% over 36 min, from 42% to 100% over 4 min, remaining isocratic at 100% for 10 min, decreasing from 100% to 15% over 1 min; remaining isocratic at 15% for 9 min); injection volume of 10 μl; UV detection at 220 nm. (2) MS: Agilent 1100 series LC/MSD trap (Agilent Technologies, Basel, Switzerland); ESI (ElectroSpray Ionization) source in negative mode; modified ionization parameters (dry temperature at 325°C, nebulizer (N_2) at 45.0 Ψ, dry gas at 8 l/min, capillary voltage at 3.5 kV); ion optic voltages with a RF amplitude of 150 Vpp, −160 V at the capillary exit and −40 V at the skimmer; specific trap parameters (trap drive at 74 V, scan from 200 to 1,200 *m*/*z* [mass-to-charge ratio of ions], maximum accumulation time at 150 ms); multiplier voltage at 1.8 kV and dynode voltage at 7.0 kV.

Chromatograms were integrated by using the "automatic integration option" of the Agilent Chemstations software (Agilent Technologies, Basel, Switzerland). To consider only those peaks corresponding to cardenolides, peaks with a maximum falling below 219 nm or above 221 nm were excluded (the butenolide lactone ring characterizing cardenolides absorbs at 220 nm). The MS was used to corroborate this selection by the presence of fragments typical of cardenolides, which also allowed the precise identification of some of the compounds as the same or similar to those previously detected in *O. gloriosa* (Eggenberger and Rowell-Rahier 1993). Peaks in different samples with a retention time difference of less than 0.2 min were considered to be the same cardenolide, confirmed by their identical set of fragments in the MS. To standardize the minimum at which a cardenolide was considered present, the height of each peak was divided by the mean height of all peaks within each sample, and only those greater than a minimum fixed value of 0.1 were taken into consideration. The HPLC–MS analysis was performed twice for each sample, giving the same cardenolide matrix. A final presence/absence matrix of cardenolides based on their retention times in all sampled sites was obtained after removing invariable peaks. Based on these data, a similarity matrix was created by computing the Jaccard indexes between sites using the "R Package for Multivariate and Spatial Analysis" version 4 (Casgrain and Legendre 2001). To later compare matrices at the individual level (as genetic distance matrices were computed at the individual level and not at the population level, see below), the "population information" was duplicated among individuals from the same sites to build an individual-based distance matrix in which the two individuals from each site showed the same cardenolide and environmental distance patterns.

DNA extraction, PCR amplification and gene sequencing Total genomic DNA was extracted from two individuals per site, using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). Extraction was performed using 4–6 legs of each individual. Three mtDNA genes were amplified using insect universal primers: *16s ribosomal RNA* (LR-N-13398 and LR-J-12883 from Simon et al. 1994), partial *cytochrome oxidase I* (*COI*) (C1-J-1751 and C1-N-2191 from Simon et al. 1994) and partial *cytochrome oxidase II* (*COII*) (modTL2-J-3037 and modC2-N-3661 from Mardulyn et al. 1997). Gene amplification was carried out in a standard 30 μl PCR reaction including: 3 μl of 10X PCR buffer (Promega, Madison, WI, USA), 3 μl of a $MgCl_2$ solution (25 mM), 3 μl of dNTPs (1.5 mM), 0.5 μl of forward and reverse primers (10 mM), 0.3 μl of Taq DNA polymerase (Promega, Madison, WI, USA), 3 μl of extracted DNA, all made up to 30 μl with purified MilliQ water. The PCR reactions were run in a TGradient thermocycler (Biometra, Goettingen, Germany) with the following program: initial denaturation at 93°C for 1 min 30 s; 35 cycles comprising

denaturation steps at 93°C for 1 min 30 s, annealing steps at 45°C (*16s rRNA*, *COI*) or at 53°C (*COII*) for 1 min, extension steps at 72°C for 2 min; and final extension at 72°C for 8 min. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Sequencing was carried out by Macrogen (Seoul, South Korea). They performed sequencing with both forward and reverse primers with the three genes under BigDyeTM terminator cycling conditions, purifying the reacted products by using ethanol precipitation, and running them using an Automatic Sequencer 3730XL (Applied Biosystem, Foster City, USA).

Phylogenetic reconstruction and genetic distance matrix 16s rRNA, COI, and COII sequences were reconstituted based on the forward and reverse sequences using Chromas Pro 1.33 (Technelysium, Helensvale, Australia). For *16s rRNA*, alignment was carried out with ClustalW 1.4 (Thompson et al. 1997). For *COI* and *COII*, alignment was trivial as all sequenced fragments were of the same size. We performed partitioned Bayesian analyses (Jordal and Hewitt 2004; Nylander et al. 2004) on the combined data set of the sequences for the three genes. All analyses were performed on a computer cluster at the Bioportal, University of Oslo, using MrBayes version 3 (Ronquist and Huelsenbeck 2003). For each defined partition (one partition per gene sequenced), the best-fit substitution model was determined by using Modeltest 3.06 (Posada and Crandall 1998) through the Akaike information criterion (AIC) selection model (Posada and Buckley 2004), using separate starting NJ Jukes–Cantor-based trees for each partition. In MrBayes, distinct parameters were, therefore, used *a priori* for each partition defined under the previously Modeltest-determined best-fit substitution models. Four Metropolis-coupled chains with incremental heating in four distinct runs of 1,000,000 generations were performed, discarding the first 200,000 generations (burn-in) in each. One tree was saved every 100 generations, and a consensus tree was built by adding all compatible groups. Based on this consensus tree, additive tree distances (Sattath and Tversky 1977) between units were computed using Darwin version 5.0 (Perrier et al. 2003) to infer a genetic distance matrix between individuals computed from the partitioned multigene Bayesian analysis. To our knowledge, only Bayesian methods allow genes to be considered to be evolving independently (i.e., with different evolutionary models) in one single analysis. The phylogenetic hypothesis is then used to determine a multipartition mtDNA distance matrix. This would not have been possible using classical distance, parsimony, or likelihood methods, which do not allow partitioned analyses.

Combined analyses Distance matrices based on (1) cardenolide composition, (2) genetic distances, (3) habitat distances, and (4) host-plant distances were compared by performing Mantel tests (a total of six tests) using the R package followed by Bonferroni correction.

For the two comparisons that were significant (cardenolide composition with genetic distance and with host-plant use), tests were made on the individual compounds and hosts. The Mantel test between host-plant distance and cardenolide distance was repeated while sequentially removing individual host plants and also with each plant species alone to test which were important. To look at the role of individual cardenolides, an ANOVA was performed in JMP (2003) with pairwise genetic distance as the response and each cardenolide peak as a factor (1 when shared and 0 when not). A similar analysis was carried out with host-plant use as the response, based on the Jaccard index between populations. Furthermore, a regression was performed to test if the number of peaks in the cardenolide blend was related to the number of host plants within a site.

Finally, UPGMA analysis (complemented with NJ and maximum likelihood analyses) was used to construct a tree based on the cardenolide composition distance matrix to investigate the overall pattern of grouping among sites. We used the "R Package for Multivariate and Spatial Analysis" (Casgrain and Legendre 2001) for UPGMA tree reconstruction, the restdist module (from the PHYLIP package; Felsenstein 2004) for Nei–Li-based NJ reconstruction, and the restML module (from the PHYLIP package; Felsenstein 2004) for maximum likelihood analysis.

Results

Variability in the genetic and chemical data Our analysis revealed a total of 50 cardenolides, of which 14 were found in all populations. Two substances were detected in only one population, whereas four were present in all populations except one. The number of cardenolides per population varied between a maximum of 42 (FR13) and a minimum of 19 (FR9) (Table 1). However, 2/3 of the populations contained between 30 and 38 cardenolides. On average, pairs of populations shared 66% of their cardenolides, with a maximum (85%) shared between IT1 and VS8 and a minimum (41.8%) between FR9 and FR13.

The genetic analysis yielded a total of 1,510 bp among the three mtDNA genes: 503 bp for *16s rRNA* (8 informative sites among 14 polymorphic sites), 447 bp for partial *COI* (34 informative sites among 44 polymorphic sites), and 560 bp for partial *COII* (30 informative sites among 43 polymorphic sites). This amount of variation—with the proportion of polymorphic sites ranging from 2.5% (ribosomal RNA) to 10% (coding genes)—is common in insect mtDNA at the intraspecific level (Simon et al. 1994). Sequences were deposited in GenBank (accession numbers EF635121 to EF635217). The best-fit substitution models for each of the three genes were determined by Modeltest as follows. *COI*: transitional model (TIM) with gamma parameter and proportion of invariable sites; *COII*: transversional model (TVM) with gamma parameter and proportion of invariable sites; *16s rRNA*: transversional model with equal base frequencies (TVMef) with proportion of invariable sites. The phylogenetic tree obtained by Bayesian inference (not shown) presented a log(likelihood) equal to −3,817.8 and corresponded to the following parameters: (1) *COI* transition rates: Rmat(A–C)=0.126168, Rmat(A–G)=0.2801, Rmat(A–T)=0.0320, Rmat(C–G)=0.1747, Rmat(C–T)=0.3599, Rmat(G–T)=0.0271; *COI* estimated base frequencies: p(A)=0.4239, p(C)=0.1299, p(G)=0.0945, p(T)=0.3517; *COI* gamma parameter: shape=0.0547; *COI* proportion of invariable sites: 0.8636; (2) *COII* transition rates: Rmat(C–G)=0.0349, Rmat(C–T)=0.3477, Rmat(G–T)=0.0302; *COII* estimated base frequencies: p(A)=0.3049, p(C)=0.2005, p(G)=0.1305, p(T)=0.3642; *COII* gamma parameter: shape=14.7693; *COII* proportion of invariable sites: 0.7671; (3) *16s rRNA* transition rates: Rmat(A–C)=0.0517, Rmat(A–G)=0.3449, Rmat(A–T)=0.1454, Rmat(C–G)=0.0116, Rmat(C–T)=0.4303, Rmat(G–T)=0.0161; *16s rRNA* proportion of invariable sites: 0.7216.

Geographical variation The UPGMA clustering of populations based on the Jaccard matrix of cardenolides shows strong, but not perfect, geographical structure (Fig. 1). There was broad-scale east–west division: Balkan and eastern Alpine populations (HR2, HR4, Slo2, Slo9, Slo10) fell with those from the Rhone Valley in the Swiss Alps (VS4, VS5, VS6, VS7), whereas populations from the south–west Alps (FR13, FR14, IT1, VS8)

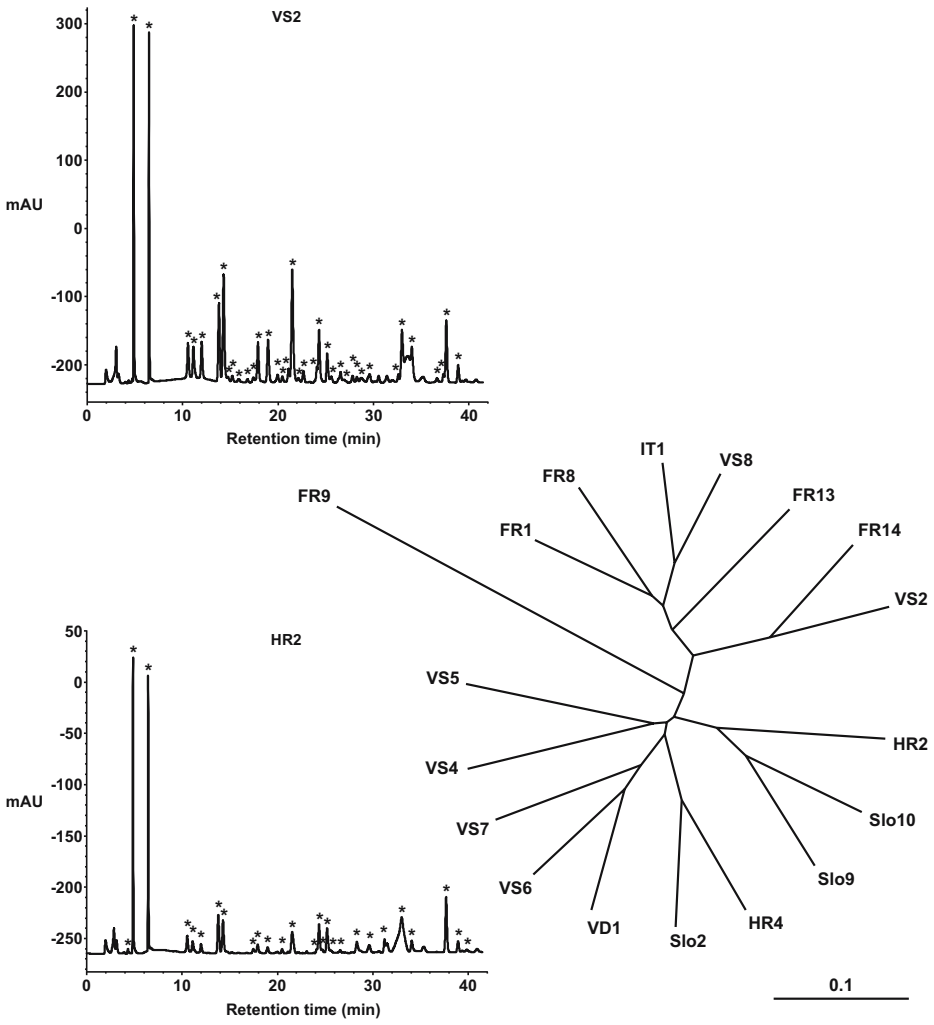


Fig. 1 HPLC traces of defensive secretion of *O. speciosa* from two populations (with each cardenolide peak shown by an *asterisk*) and UPGMA clustering of populations based on their cardenolide composition. Abbreviations for the populations are the same as in Table 1

clustered together with one population from the Jura (FR1) and one from the Massif Central (FR8). Some populations fell in unexpected positions (VD1 and VS2), and FR9 was found at the end of a long branch. The main two-cluster topology remains when performing other analyses such as Nei–Li-based NJ and maximum likelihood (not shown) with the exception of the position of FR9, which sometimes switches between clusters.

Influence of environmental factors and genetics on the overall cardenolide blend Of the six Mantel tests, only that between cardenolide distance and host-plant distance and that between cardenolide distance and genetic distance were significant after Bonferroni's correction (Table 2). The significant effect of host plant on cardenolide composition was not the result of a correlation between the number of host plants and the number of

Table 2 Results of Mantel tests for the pairwise distance matrices

	Cardenolides	Habitat	Host Plants	Genetic Distance
Cardenolides		P=0.075	P=0.006[a]	P=0.005[a]
Habitat	r=0.165		P=0.021	P=0.108
Host plants	r=0.299[a]	r=0.222		P=0.033
Genetic distance	r=0.224[a]	r=0.076	r=0.128	

[a] Significant after Bonferroni's correction.

cardenolides in the blend (linear regression, $F_{1,\ 16}$=0.736, P=0.404). When plants were tested individually, only *Heracleum sphondylium* had a significant Mantel correlation with the cardenolide composition (r=0.166, P=0.049), whereas the correlations for *Angelica sylvestris* and *Seseli libanotis* were marginally nonsignificant (Table 3). The importance of these three plants was confirmed by removing them sequentially from the overall Mantel test between host plant and cardenolide composition. The correlation remained significant when one or two of these plants were removed, but disappeared when removing all three.

Influence of genetics and host plants for each cardenolide separately Nine cardenolides were significantly correlated with genetic distance (Table 4). In contrast, despite the fact that the whole blend of cardenolides was strongly correlated with host-plant use, there were no such correlations at the level of single cardenolides. Nevertheless, simply observing the presence/absence of cardenolides in populations revealed one pattern. Three cardenolides (RT=17.9, 19.0 and 21.6) were present in all sites except one, Puy de Dôme (FR9). This was the only population in our study where *O. speciosa* feeds on *S. libanotis*, and also the only population that lacks both *H. sphondylium* and *P. ostruthium* (Table 1).

Discussion

Autogenously synthesized chemical defense in *O. speciosa* showed considerable geographic variation: 36 out of 50 cardenolides varied in this sample of 18 populations, and no two populations showed the same cardenolide profile. Some of this variation is associated with genetic divergence, with a significant relationship for the overall blend and for nine of the 36 variable cardenolides when tested separately. This is clearly not evidence

Table 3 Results from Mantel correlations between the cardenolide distance matrix and matrices based on individual host plants

Host Plant	Number of Sites Where Present	r
Angelica sylvestris	3	0.264[†]
Anthriscus sylvestris	9	0.016
Chaerophyllum villarsii	3	0.093
Heracleum sphondylium	12	0.166*
Laserpitium latifolium	2	0.083
Peucedanum ostruthium	10	0.060
Seseli libanotis	1	0.557[†]

*P<0.05

[†] 0.05<P<0.10

Table 4 Details of the 50 cardenolides and results from the ANOVA on the pairwise genetic distance between populations with individual cardenolides treated as explanatory factors

Cardenolide (retention time)	Present in *N* Sites	*F* Value	*P* Value
4.3	7	2.68	0.103
4.8	18†	–	–
6.5	18†	–	–
10.6	18†	–	–
10.8	2	0.43	0.515
11.1[a]	16	7.39	0.007
11.3	13	0.01	0.908
12.0	18†	–	–
12.8	1	12.38	<0.001*
13.8	18†	–	–
14.3[b]	18†	–	–
14.7	5	0.01	0.939
15.2	9	0.04	0.840
15.9	3	2.50	0.115
16.8	3	<0.01	0.980
17.6	18†	–	–
17.9	17	20.01	<0.001*
19.0[c]	17	20.01	<0.001*
19.9	12	0.01	0.936
20.5	16	0.08	0.779
21.1	10	1.78	0.183
21.6	17	20.01	<0.001*
22.2	8	0.30	0.587
22.6	7	11.97	<0.001*
23.1	8	0.12	0.733
23.6	2	13.65	<0.001*
24.0	14	2.67	0.103
24.4[d]	18†	–	–
24.7	5	5.18	0.023
25.2	18†	–	–
25.6[e]	10	22.92	<0.001*
26.6	17	2.77	0.097
27.1	8	1.20	0.274
27.8	11	0.05	0.817
28.3	16	0.70	0.405
28.9	11	3.29	0.071
29.6	18†	–	–
30.2	4	2.02	0.156
31.2	9	14.89	<0.001*
32.0	12	0.11	0.743
32.6	12	4.00	0.046
33.0[f]	18†	–	–
34.1[g]	18†	–	–
36.6	7	1.87	0.173
37.4	8	1.28	0.259
37.7[h]	18†	–	–
38.9[i]	18†	–	–
39.3	3	3.55	0.060

Table 4 (continued)

Cardenolide (retention time)	Present in *N* Sites	*F* Value	*P* Value
39.9	6	15.91	<0.001*
40.4	1	2.35	0.126

[a] Monomorphic cardenolides: these compounds were found in all populations

[b] Tetrahydroxylated aglycon-hexopyranoside

[c] Significant after Bonferroni's correction

[d] Periplogenin-3-*O*-[β-D-xylopyranosyl-(1→4)-β-D-allopyranoside]

[e] Tetrahydroxylated aglycon-acetyl-hexopyranoside

[f] Didehydroperiplogenin-3-*O*-β-D-allopyranoside

[g] Periplogenin-3-*O*-β-D-allopyranoside

[h] Didehydroperiplogenin-*O*-acetyl-β-D-allopyranoside

[i] Periplogenin-3-*O*-acetyl-β-D-allopyranoside

[†] Didehydrodigitoxigenin-3-*O*-[-*O*-acetyl-β-D-xylopyranosyl-(1→4)-*O*-acetyl-β-D-allopyranoside]

[*] Digitoxigenin-3-*O*-[-*O*-acetyl-β-D-xylopyranosyl-(1→4)-*O*-acetyl-β-D-allopyranoside]

of a direct link between divergence at mitochondrial DNA and shifts in chemical defense. Instead, mtDNA serves as a marker of the level of divergence (assuming that mtDNA variation reflects the overall genomic variation) between what are probably strongly isolated populations (Margraf et al. 2007). More genetically divergent populations have probably experienced longer or stronger isolation and, therefore, a greater opportunity for the composition of their defense secretions to diverge by natural selection or genetic drift.

The host plant, a key element of the environment for herbivorous insects, was also important for the expression of some cardenolides. Such an influence of host-plant use is new and intriguing for insects that produce substances *de novo*. In other systems with sequestered compounds, there is a direct link between the host plants on which the insect feeds and the chemical profile of their defense secretions (Malcolm et al. 1989; Kopf et al. 1998; Moranz and Brower 1998; Wahlberg 2001; Verdon et al. 2007). For autogenously produced cardenolides, however, the relationship was unexpected. It is probably explained by the fact that cardenolides are synthesized from plant sterols (and possibly from other compounds such as sugars) that vary between plant species and provide an initial source of variation.

Those cardenolides that could be explained neither by genetic inheritance nor by the tested environmental factors must be the consequence of other sources of variation, such as geographical differences in plant chemistry, phenotypic plasticity, cyclic variation (e.g., seasonal cycles [Fordyce et al. 2005]), other biotic or abiotic environmental pressures (e.g., temperature), or simple random processes. Antagonistic or synergistic interactions between genetic and environmental factors could also explain the fact that many substances did not show a simple correlation with single phylogenetic or environmental distances.

The evolutionary forces driving this intraspecific polymorphism are clearly complex, involving both genetic and environmental influences. Divergence among populations is in part influenced directly by host-plant use, but may also be under natural selection depending on the level of predation or type of predators encountered. Genetic drift is likely to contribute, perhaps if the overall level of cardenolide defense is fixed but the individual

components are free to vary. There also seem to be constraints acting, with 14 of the components invariable across populations.

The number of cardenolides found within each population was remarkable, with between 19 and 42 compounds present. These secretions were pooled samples from 5 to 10 beetles, but because most of the variation in defense within *Oreina* populations is quantitative rather than qualitative (Eggenberger and Rowell-Rahier 1992), individuals are likely to possess the full blend of cardenolides typical of a population. The compounds varied in the number of sugars and the identity of both the sugar and aglycone components (Table 1). The complexity may have a functional explanation, if a blend is more efficient in promoting predator learning or deterrence. It could also simply be a result of the process of synthesis, with a mixed diet providing variation in the initial sterols and the inclusion of by-products and precursors in the secretion further increasing its diversity.

The geographical coherence of cardenolide composition was also striking. Populations were clustered into broadly eastern and western groups (consisting of an E-Alps/Balkan/Rhone valley group and a SW-Alps/Jura/Massif Central group, Fig. 1). This is not a result of host-plant use through host shifts or intraspecific variation in host chemistry, for the plants show no such structuring and are thoroughly mixed throughout the distribution (Table 1 with the one exception of *Seseli libanotis* found only at FR9). It is also not related to differences in the mean altitude of the two groups (Table 1), and so probably not influenced by current climatic conditions. It may be a product of the shared evolutionary history of populations, with two main clusters possibly corresponding to two different Pleistocene refugia and subsequent migration. Similar large-scale structuring is seen in other alpine insects and plants (Schönswetter et al. 2005; Schmitt et al. 2006; Margraf et al. 2007), and future phylogenetic work will test if the different lineages within *O. speciosa* present a biogeographic signal compatible with the history of climatic oscillations in Europe. The revelation of this east/west split shows the value of extending chemosystematics to the use of chemical markers at the intraspecific level. Although chemical defense based on cardenolides is typical of several lineages within the subtribe Chrysolinina to which *Oreina* belongs, there is sufficient variation within a species to group populations and investigate the interaction between their current ecology and evolutionary history.

Acknowledgments The authors wish to thank Matthias Borer for help during fieldwork, Eliane Abou-Mansour for help with chemical analysis, and the National Centre of Competence in Research (NCCR) Plant Survival for funding chemical and genetic analyses.

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J Chem Ecol (2007) 33:2025–2027
DOI 10.1007/s10886-007-9381-3

ERRATUM

Genetic and Environmental Sources of Variation in the Autogenous Chemical Defense of a Leaf Beetle

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Published online: 24 October 2007
© Springer Science + Business Media, LLC 2007

The footnote to Table 4 does not correspond to the superscripts in the body of the table. The correct Table 4 appears below.

The online version of the original article can be found at doi:10.1007/s10886-007-9351-9.

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Table 4 Details of the 50 cardenolides and results from the ANOVA on the pairwise genetic distance between populations with individual cardenolides treated as explanatory factors

Cardenolide (Retention Time)	Present in *N* Sites	*F* Value	*P* value
4.3	7	2.68	0.1026
4.8	18†	–	–
6.5	18†	–	–
10.6	18†	–	–
10.8	2	0.43	0.5146
11.1[a]	16	7.39	0.0068
11.3	13	0.01	0.9081
12.0	18†	–	–
12.8	1	12.38	0.0005 *
13.8	18†	–	–
14.3[b]	18†	–	–
14.7	5	0.01	0.9388
15.2	9	0.04	0.8403
15.9	3	2.50	0.1146
16.8	3	<0.01	0.9795
17.6	18†	–	–
17.9	17	20.01	<0.0001 *
19.0[c]	17	20.01	<0.0001 *
19.9	12	0.01	0.9359
20.5	16	0.08	0.7785
21.1	10	1.78	0.1828
21.6	17	20.01	<0.0001 *
22.2	8	0.30	0.5870
22.6	7	11.97	0.0006 *
23.1	8	0.12	0.7333
23.6	2	13.65	0.0002 *
24.0	14	2.67	0.1033
24.4[d]	18†	–	–
24.7	5	5.18	0.0233
25.2	18†	–	–
25.6[e]	10	22.92	<0.0001 *
26.6	17	2.77	0.0967
27.1	8	1.20	0.2742
27.8	11	0.05	0.8166
28.3	16	0.70	0.4047
28.9	11	3.29	0.0705
29.6	18†	–	–
30.2	4	2.02	0.1562
31.2	9	14.89	0.0001 *
32.0	12	0.11	0.7425
32.6	12	4.00	0.0460
33.0[f]	18†	–	–
34.1[g]	18†	–	–
36.6	7	1.87	0.1726
37.4	8	1.28	0.2593
37.7[h]	18†	–	–
38.9[i]	18†	–	–
39.3	3	3.55	0.0601

Table 4 (continued)

Cardenolide (Retention Time)	Present in *N* Sites	*F* Value	*P* value
39.9	6	15.91	<0.0001 *
40.4	1	2.35	0.1258

† Monomorphic cardenolides: these compounds were found in all populations

*Significant after Bonferroni's correction

[a] Tetrahydroxylated aglycon-hexopyranoside

[b] Periplogenin-3-*O*-[β-D-xylopyranosyl-(1→4)-β-D-allopyranoside]

[c] Tetrahydroxylated aglycon-acetyl-hexopyranoside

[d] Didehydroperiplogenin-3-*O*-β-D-allopyranoside

[e] Periplogenin-3-*O*-β-D-allopyranoside

[f] Didehydroperiplogenin-*O*-acetyl-β-D-allopyranoside

[g] Periplogenin-3-*O*-acetyl-β-D-allopyranoside

[h] Didehydrodigitoxigenin-3-*O*-[-*O*-acetyl-β-D-xylopyranosyl-(1→4)-*O*-acetyl-β-D-allopyranoside]

[i] Digitoxigenin-3-*O*-[-*O*-acetyl-β-D-xylopyranosyl-(1→4)-*O*-acetyl-β-D-allopyranoside]

Introduction

Numerous plant traits may reduce the impact of damage by herbivores on plant fitness. These defensive traits may be expressed constitutively or may be induced following herbivory. Responses induced after damage by herbivores include changes in plant nutritional quality, the induction of defensive proteins and other allelochemicals, and the release of complex blends of volatile organic compounds (VOCs; reviewed by Karban and Baldwin 1997; Walling 2000). In many cases, VOCs may serve as cues that aid the discovery of herbivore-damaged plants by the natural enemies of those herbivores (reviewed by Arimura et al. 2005; Dudareva et al. 2006; D'Alessandro and Turlings 2006). The production of many induced VOCs is mediated, at least in part, by the jasmonic acid signaling pathway, and in many cases, VOC production similar to that caused by herbivore damage can be induced simply by exposing plants to jasmonic acid or its methyl ester, methyl jasmonate (MeJA) in the absence of herbivores (Hopke et al. 1994; Dicke et al. 1999; Degenhardt and Lincoln 2006). Induced VOC production has been studied primarily in cultivated species. Although the study of VOCs in cultivated species has logistical advantages, it is difficult to draw strong inferences on the evolution and maintenance of VOC production on cultivated species because the plants, herbivores, and their natural enemies are often far removed from their evolutionary context.

This study addressed induced VOC production in a native, undomesticated plant species. Sacred datura, *Datura wrightii* Regel (Solanaceae), produces two leaf trichome phenotypes that differ in their susceptibility to herbivorous insects. One phenotype is densely covered by short, non-glandular trichomes and feels velvety to the touch (hereafter the velvety phenotype), whereas the other phenotype is less-densely covered with glandular trichomes that secrete glucose esters and aliphatic acids, and these leaves feel sticky to the touch (hereafter the sticky phenotype; van Dam and Hare 1998a). Trichome phenotype is governed by a single locus and is inherited in a Mendelian fashion, with the allele for the glandular phenotype dominant over the nonglandular phenotype (van Dam et al. 1999). Within Southern California, the region in which this work was conducted, these two trichome phenotypes occur sympatrically, and the frequency of the sticky phenotype varies from 0 to 82% among populations (Hare and Elle 2001). For several of the herbivores that feed on *D. wrightii*, including whiteflies, leafhoppers, and flea beetles, the sticky phenotype is more resistant to attack (Hare and Elle 2002). In contrast, the chrysomelid beetle, *Lema daturaphila* Kogan & Goeden (Coleoptera: Chrysomelidae), appears unaffected by the presence of glandular trichomes (Hare and Elle 2002), and the mirid bug, *Tupiocoris notatus* Distant (Hemiptera: Miridae), specializes on the sticky phenotype (van Dam and Hare 1998b). *Lema daturaphila* is the primary herbivore of *D. wrightii* in Southern California (Hare and Elle 2002).

A number of generalist predators are associated with the herbivores of *D. wrightii*, including the damsel bug *Nabis americoferus* Carayon (Hemiptera: Nabidae; or possibly *Nabis alternatus* Parshley), the big-eyed bug *Geocoris pallens* Stål (Hemiptera: Lygaeidae), larval lacewings *Chrysoperla* spp. (Neuroptera: Chrysopidae), and the jumping spider *Habronattus pyrrithrix* Chamberlin (Araneae: Salticidae; Gassmann and Hare 2005). All of these species were less effective in suppressing *Lema daturaphila* or *T. notatus* when the herbivores were on plants of the sticky phenotype rather than on plants of the velvety phenotype (Gassmann and Hare 2005). *Lema daturaphila* is also parasitized by *Conura delumbis* (Hymenoptera: Chalcidae) and *Myiopharus infernalis* (Diptera: Tachinidae). These two widespread parasitoids have been collected from other populations and subspecies of *Lema daturaphila* (e.g., Puttler 1966), but relatively little else is known about them.

Datura wrightii shares with other solanaceous species the ability to produce elevated levels of proteinase inhibitors (PIs) and polyphenol oxidase (PPO) in response to treatment with MeJA, and induced levels of these defensive proteins in *D. wrightii* are similar to induced levels of PIs and PPO in cultivated tomato, *Lycopersicon esculentum* and potato, *Solanum tuberosum* (Hare and Walling 2006). PI and PPO levels were similarly inducible in the two trichome phenotypes of *D. wrightii*, but trichome morphology itself was not (Hare and Walling 2006).

Relatively little is known of the VOCs produced by *Datura* species. Fraser et al. (2003) reported the presence of several compounds in headspace collections from *D. wrightii* in Arizona that elicited electroantennogram activity in female *Manduca sexta*, and the presence of these compounds may serve as oviposition attractants for *M. sexta*. These compounds included (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl propionate, nonanal, decanal, linalool, phenylacetaldehyde, methyl salicylate, geranylacetone, benzyl alcohol, (*E*)-nerolidol, and six unknowns. These plants presumably expressed the velvety phenotype because the sticky phenotype is not known to occur in Arizona (Mira and Bernays 2002), and these plants also were undamaged (Fraser et al. 2003) and presumably uninduced.

The goals of this study were to better understand the induced responses of *D. wrightii*. The first objective was to determine if *D. wrightii* produces VOCs in response to damage by *Lema daturaphila* and/or treatment with MeJA. The second objective was to determine if there might be genetic variation in VOC production among a series of genetic lines of *D. wrightii*. The final objective was to determine if sibling plants within lines that differed in the expression of trichome phenotype might also differ in VOC production.

Materials and Methods

Plants Seedlings were used from eight genetic lines that originated from crosses between a randomly selected pollen donor expressing the velvety phenotype and a heterozygous pollen acceptor expressing the sticky phenotype from four local populations. The plant populations and their characteristics are fully described in Hare and Elle (2004). Two populations, Bell Canyon and Ortega Flats, were from the Santa Ana Mountains from a relatively high-rainfall "mountain" habitat of *D. wrightii*. Two other populations were from the relatively drier Riversidian Sage Scrub habitat. One of these was on the campus of the University of California, Riverside (UC), whereas the other was on an undeveloped land within the city of Moreno Valley, California (MV). Two velvety pollen donors were selected from each of the four populations. Originally, each of the eight pollen donors was crossed to each of three different sticky pollen acceptors; one acceptor was from the same population, whereas another was from a different population in the same habitat, and the third was from a population in a different habitat. The original pollen donors were propagated vegetatively and continue to be maintained in 14 l pots in the greenhouse and repotted annually. The lines used in this experiment were third-generation backcross progeny of heterozygous sticky progeny backcrossed to their original velvety pollen parent. The expected ratio of adult heterozygous sticky to homozygous recessive velvety sibs within each of the three families derived from each of the eight original pollen donors is 1:1, and sibs within families are expected to be at least 93.75% similar after three generations of backcrossing.

Two batches of seeds of third-generation backcross progeny were germinated in the spring and fall of 2006 in a greenhouse equipped with high-pressure sodium lamps providing supplemental illumination for 16 h/day so that mid-day light intensities averaged

1,250±39 μmol m^{-2} s^{-1} PAR illumination (N= 5 readings) at plant height. Greenhouse temperatures ranged between 15° and 35°C. Plants were maintained under these conditions for about 3 months until trichome differentiation was complete (van Dam et al. 1999). One group of plants was used to determine if feeding by *Lema daturaphila* induced VOCs in *D. wrightii*, whereas the second was used to determine if treatment with MeJA also induced VOCs.

Collection of Volatiles Although it would have been simpler to collect the volatiles from detached leaves, Schmelz et al. (2001) showed that detached leaves of maize emitted greater quantities and different blends of VOCs than did leaves attached to their plants. To avoid such potential artifacts, volatiles were collected nondestructively from intact plants. Plants were 30–50 cm tall and had produced 10–15 leaves. After plants had completed trichome differentiation, they were brought to the laboratory and nondestructively aerated for 24 h in a plant growth room [16:8 photophase/scotophase, 26°C day and 20°C night temperatures with light intensity of 293 (±29 SE, N=5) μmol m^{-2} s^{-1} PAR illumination at plant height]. Aeration chambers were made from polyester cooking bags (unprinted 45× 55 cm bags, Terinex, Bedford, England) largely as described by Stewart-Jones and Poppy (2006). Swagelok® fittings with polytetrafluoroethylene (PTFE) ferrules were inserted in two slits in the bags, one for an air inlet and the other for an air outlet. Fittings were sealed to the bags with stainless-steel hose clamps. Bags were then flushed with about 40 l of compressed air (Medical grade) that was filtered through activated charcoal. Preliminary studies showed virtually no residual volatile compounds from new, flushed bags.

A bag was placed over a whole plant, excluding senescing leaves, and secured with a twist-tie around the stem. Intake and exhaust flow rates were regulated with flow meters (Aalborg, Orangeburg, NY, USA). PTFE tubing was used upstream of the adsorbent trap, and polyvinylchloride (PVC) tubing was used downstream. Compressed air, as above, flowed into the bag at 600 ml/min. After the bag was loosely inflated, air was exhausted through an adsorbent trap at the same rate with a house vacuum. VOCs were adsorbed on traps made of glass tubing and filled either with activated charcoal (10 mg) or Super-Q (25 mg, Alltech, State College, PA, USA). Preliminary experiments showed similar performance of the two adsorbents. At the termination of the aeration, traps were wrapped in aluminum foil, labeled, and stored at −20°C for extraction later that day.

Sampling procedures were developed to give highest priority to detect differences in VOC production between trichome phenotypes. Groups of four plants (two pairs of sticky and velvety sibs) were aerated at a time. The experiment was arranged in three blocks, with each block containing one of the three families from each of the original eight pollen donors, and each block of plants was aerated in succession. Within blocks, families from different pollen donors were selected at random for aeration. These procedures were adopted to avoid confounding potential variation in the inducibility of plants from different genetic lines with potential age-related differences over the 24-day VOC collection period.

Insect Damage Treatment After plants were aerated the first time, they were placed in a single cage and 15–20 second, and third instars of *Lema daturaphila* were placed on each of the plants. Larvae were allowed to feed for 24 h, then the leaf area removed was assessed (Hare and Elle 2002), and larvae were removed. Plants were returned to a separate greenhouse with similar conditions as the first. After an additional 48 h, plants were returned to the laboratory and aerated once again for 24 h with clean bags and traps. Preliminary studies showed relatively little VOC induction until 2–3 days after insect damage. To control for potential variation in the number of leaves aerated, aerated leaves

were removed from the plant, dried to a constant weight at 80°C, and the dry weight was recorded. This information was used to calculate the release rate of VOCs per unit of leaf mass (see below). Care was taken to ensure that exactly the same leaves were aerated before and after treatment. A total of 48 plants (8 genetic lines×3 families within each line×2 trichome phenotypes) were aerated before and after insect damage.

MeJA Treatment After these plants were aerated before MeJA treatment, they were returned to a separate greenhouse with similar rearing conditions and treated with a suspension of 8 mM MeJA (Bedoukian Research) in 0.1% Tween 20 (Sigma). This rate is typical of those used in previous studies (e.g., Boughton et al. 2005), and preliminary studies showed that this rate provided a response similar to that caused by insect damage (see "Results") without causing any visible injury to plants. Leaves of plants were sprayed just to run-off (10–15 ml per plant). Plants remained in this greenhouse for 72 h, after which they were returned to the laboratory and aerated once again for 24 h by using fresh bags and traps. At the end of the aeration, traps were removed and extracted, and leaves were collected, dried, and weighed as above. Only 44 plants were aerated because sibs from 2 families (out of 24) grew atypically slowly and were not sampled.

A slightly different collection apparatus was used to sample quantities of (*E*)-β-caryophyllene produced by the original male parents of test plants. For these plants, volatiles were collected from plants in the greenhouse with methods similar to those that Kessler and Baldwin (2001) used to collect volatiles from plants in the field. Rigid polyethylene terephthalate extraction chambers were made from soft drink cups (Solo Cup, Highland Park, IL, USA., model TP16 cups and DL626 lids) that enclosed a single full-sized leaf of a plant. Ambient air was drawn through the cups for 24 h with a 12-V battery-powered vacuum pump (Gast Mfg., Benton Harbor, MI, USA) powered by a deep-cycle marine storage battery, and the volatiles were adsorbed on a Super-Q trap, as above. A manifold was used to draw air through 16 lines at a time at a mean (±SE) flow rate of 680±23 ml/min per line. Fifteen plants were sampled at a time, and the 16th line was connected to an adsorbent trap attached to an empty chamber ('blank') to quantify and control for ambient volatiles in the greenhouse. Extractions were made on five clonal individuals of each original pollen donor before and after treating the whole plant with MeJA, then waiting 72 h as above. In each case, a young but full-sized leaf was extracted, and the sampled leaf was subsequently removed, dried, and weighed.

Volatiles Analysis Volatiles were eluted from the traps with CH_2Cl_2 that contained 4 ng/μl of *n*-bromoheptane as an internal standard. Activated charcoal traps were eluted with 60 μl, whereas the Super-Q traps were eluted with 150 μl. Extracts were stored in 200 μl vials sealed with PTFE closures and stored at −20°C. One micro liter of each extract was analyzed on a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies, Avondale, PA, USA) with a flame ionization detector with a DB-5 column (60 m, 0.25 mm ID, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). Injector and detector temperatures were 200 and 250°C, respectively. Injections were made in splitless mode with a column flow of 25 cm/s with helium as the carrier gas. The oven was programmed from 40 to 250°C at 5°C/min with a hold at the final temperature for 5 min. To confirm identifications, selected samples were analyzed with a Hewlett-Packard 6890 GC coupled to a Hewlett-Packard 5973 mass selective detector, and the mass spectra and retention times of compounds were matched with those of authentic standards. Most standards were obtained from Sigma-Aldrich and subsidiaries. Samples of (*E*,*E*)-4,8,12 trimethyl-1,3,7,11 tridecatetraene (hereafter TMTT), (*E*)-4,8,dimethyl-1,3,7 nonatriene (hereafter DMNT), and (*E*)-β-ocimene were kindly provided by Dr. J. G. Millar. Peak quantification was made by

using Agilent ChemStation software, and the detected volatiles were quantified based on comparison of their peak height with those of the internal standard. Quantities of volatiles released are expressed in units of ng per mg leaf (dry wt) per hour.

Statistical Analyses Because volatiles were collected from plants before and after feeding by insects or application of MeJA, the appropriate analysis was a repeated measures analysis with each plant as a 'subject.' Such an analysis provides relatively good control for variation within treatments to detect differences between treatments. Because the response variables are likely correlated, it is inappropriate to analyze each by univariate methods. Instead, a 'multivariate analysis of variance (MANOVA)-protected ANOVA' approach was used in which the group of VOCs was first analyzed by MANOVA, and then ANOVAs were carried out on the individual VOCs only if the MANOVA was statistically significant (Scheiner 2001). Repeated-measure MANOVAs were carried out with trichome phenotype, genetic line, and treatment and their two-way and three-way interactions as fixed effects and plants within trichome phenotype by genetic line combinations as the repeated, random 'subjects' effect using PROC GLM of SAS (SAS Institute 2000). Because of the unequal sample sizes in the MeJA experiment where two of the lines had only two families instead of three, the univariate analyses were carried out by using a restricted maximum likelihood method (PROC MIXED of SAS), which is more robust than PROC GLM with unequal sample sizes. The (log +1) transformation and the arcsine square root transformation were applied to quantitative and proportional data, respectively, so that the residuals would be normally distributed.

Results

Seventeen compounds generally were found in plants induced by insect feeding or treatment with MeJA. Most of these compounds were also found in the volatiles of uninduced plants but at lower concentrations (Table 1). By far, the most abundant compound in plants induced either by feeding by *Lema daturaphila* or MeJA treatment was (*E*)-β-caryophyllene. Although methyl salicylate, geranylacetone, phenylacetaldeyde, and benzyl alcohol were reported from the volatiles of *D. wrightii* in Arizona (Fraser et al. 2003), these compounds were detected, at best, only occasionally and in trace amounts in this study. A number of hexenyl esters also occurred in a few samples in trace amounts. Methyl jasmonate and jasmone were detected from MeJA-treated plants, but not from insect-damaged plants. Because these two compounds likely are artifacts of MeJA treatment, they were deleted from all analyses. With the exception of (*Z*)-3-hexenyl acetate, none of the 'green leaf volatiles' was detected in either experiment. Most of these compounds usually are produced immediately after damage occurs, and their production may have ceased before aerating plants 48 h after insect damage had been terminated.

Larvae of *Lema daturaphila* removed about 10–15% of available leaf tissue during the 24-h feeding period. The quantities of leaf area consumed were ignored in the calculation of VOC production in undamaged plants. This is likely a conservative error in the analysis because it would slightly overestimate the VOC production of undamaged plants, thus making it more difficult to detect increases in VOC production due to insect feeding. Feeding damage was sufficient to cause an increase in the quantities of VOCs overall (MANOVA, Wilks' λ=0.096056, F=10.00, ndf=16, ddf=17, P<0.001). VOC production also varied among genetic lines, (MANOVA, Wilks' λ=0.000069, F=3.64, ndf=112, ddf=119.6,

Table 1 Production of individual volatile compounds [least-squares means ng mg (dry wt)$^{-1}$ HR^{-1}±SE] of sticky and velvety sibs before and after damage *by Lema Daturaphila* or treatment with MeJA

Compound	Insect-damaged plants		MeJA-treated plants	
	Sticky phenotype	Velvety phenotype	Sticky phenotype	Velvety phenotype
(*Z*)-3-hexenyl acetate				
Before	3.2±15.8	2.6±15.8	2.5±1.0	1.3±1.0
After	41.8±15.8	56.8±15.8	4.8±1.0	3.9±1.0
Limonene				
Before	0.6±0.3	0.4±0.3	0.6±0.3	0.5±0.3
After	2.1±0.3	2.0±0.3	1.5±0.3	1.5±0.3
(*E*)-β-Ocimene				
Before	4.1±11.1	2.7±11.1	0.9±1.4	0.2±1.4
After	37.2±11.1	50.3±11.1	7.8±1.4	9.5±1.4
Linalool				
Before	1.9±2.9	0.8±2.9	0.1±1.0	0.1±1.0
After	7.2±2.9	6.8±2.9	0.5±1.0	2.5±1.0
Nonanal				
Before	0	0	0.0±0.2	0.2±0.2
After	0	0	0.1±0.2	0.3±0.2
DMNT				
Before	18.5±28.2	1.3±28.2	0.4±1.1	0.2±1.1
After	52.4±28.2	54.1±28.2	2.3±1.1	1.8±1.1
Decanal				
Before	2.4±2.8	2.4±2. 8	0.4±0.4	0.1±0.4
After	5.6±2.8	5.7±2.8	0.7±0.7	1.2±0.4
Unknown sesquiterpene 1				
Before	0.2±0.9	0.4±0.9	0.1±0.1	0.1±0.1
After	2.8±0.9	1.5±0.9	0.2±0.1	0.3±0.1
Unknown sesquiterpene 2				
Before	1.9±0.6	2.4±0.6	1.5±0.4	1.2±0.4
After	2.6±0.6	2.4±0.6	3.2±0.4	2.9±0.4
(*E*)-β-Caryophyllene				
Before	12.3±24.8	10.6±24.8	48.1±34.6	17.9±34.6
After	111.6±24.8	204.3±24.8	344.1±34.6	317.4±34.6
α-Bergamotene[a]				
Before	0.1±1.0	0.1±1.0	0.1±1.8	0.1±1.8
After	3.4±1.0	4.9±1.0	2.2±1.8	6.4±1.8
α-Humulene				
Before	0.6±1.2	0.2±1.2	1.8±1.4	0.5±1.4
After	5.3±1.2	8.6±1.2	13.6±1.4	12.3±1.4
Unknown sesquiterpene 3				
Before	0.6±0.5	0.9±0.5	2.0±0.4	1.3±0.4
After	1.6±0.5	1.2±0.5	3.1±0.4	3.0±0.4
β-Selinene				
Before	11.6±29.8	4.4±29.8	1.2±2.6	0.1±2.6
After	93.8±29.8	119.7±29.8	6.8±2.6	14.3±2.6
Nerolidol				
Before	0.1±0.5	0.0±0.5	0.3±0.2	0.2±0.2
After	0.0±0.5	1.1±0.5	0.2±0.2	0.6±0.2
TMTT				
Before	0.9±1.8	1.9±1.8	2.4±2.1	1.9±2.1
After	3.9±1.8	8.8±1.8	7.5±2.1	10.3±2.1

Table 1 (continued)

Compound	Insect-damaged plants		MeJA-treated plants	
	Sticky phenotype	Velvety phenotype	Sticky phenotype	Velvety phenotype
Unknown sesquiterpene 4				
Before	7.2±1.7	13.3±1.7	4.7±1.9	4.8±1.9
After	8.6±1.7	7.6±1.7	5.4±1.9	4.5±1.9

[a] Tentative identification based upon mass spectrum

$P<0.001$) and due to the interaction between insect feeding treatment and genetic line (MANOVA, Wilks' $\lambda=0.002718$, $F=1.59$, *ndf*=112, *ddf*=119.6, $P=0.006$). Thus, the change in VOC production due to insect damage itself varied among genetic lines. Feeding by *Lema daturaphila* increased the production of the individual compounds, (*Z*)-3-hexenyl acetate ($F_{1,\ 32}=25.53$, $P<0.001$), limonene ($F_{1,\ 32}=22.92$, $P=0.001$), (*E*)-β-ocimene ($F_{1,\ 32}=71.84$, $P<0.001$), linalool ($F_{1,\ 32}=7.79$, $P=0.009$), DMNT ($F_{1,\ 32}=8.84$, $P=0.006$), unknown sesquiterpene 1 ($F_{1,\ 32}=4.43$, $P=0.043$), (*E*)-β-caryophyllene ($F_{1,\ 32}=142.8$, $P<0.001$), α-bergamotene ($F_{1,\ 32}=34.41$, $P<0.001$), α-humulene ($F_{1,\ 32}=60.51$, $P<0.001$), β-selinene ($F_{1,\ 32}=56.38$, $P<0.001$), and TMTT ($F_{1,\ 32}=8.63$, $P=0.006$, Table 1). The quantities of (*Z*)-3-hexenyl acetate ($F_{7,\ 32}=4.21$, $P=0.002$), limonene ($F_{7,\ 32}=2.37$, $P=0.045$), DMNT $F_{7,\ 32}=6.43$, $P<0.001$), unknown sesquiterpene 2 ($F_{7,\ 32}=4.76$, $P<0.001$), (*E*)-β-caryophyllene ($F_{7,\ 32}=6.98$, $P<0.001$), α-humulene ($F_{7,\ 32}=4.18$, P=0.002), and β-selinene ($F_{7,\ 32}=3.37$, $P=0.008$) also differed overall among genetic lines. Interactions between genetic line and insect damage were significant for (*E*)-β-ocimene ($F_{7,\ 32}=2.32$, $P=0.049$), α-humulene ($F_{7,\ 32}=2.67$, P=0.027), and unknown sesquiterpene 4 ($F_{7,\ 32}=2.52$, $P=0.034$), indicating that the increase in the production of these compounds after insect damage varied among genetic lines.

The patterns of variation may be summarized simply by focusing upon the proportion of (*E*)-β-caryophyllene, the most abundant compound, and total VOC production. The percent of the total blend that was (*E*)-β-caryophyllene varied due to treatment ($F_{1,\ 32}=54.21$, $P<0.001$) and genetic line ($F_{7,\ 32}=3.42$, $P=0.008$). After insect damage, the proportion of (*E*)-β-caryophyllene ranged from a low of 17.9% in the UCV9 line to a high of 59.2% in the OFV6 line (Fig. 1). Total VOC production differed significantly only due to insect treatment and genetic line ($F_{1,\ 32}=84.7$, $P<0.001$ and $F_{7,\ 32}=2.59$, $P=0.031$, Fig. 2). Total volatile production increased after insect damage by 3.9-fold in the OFV6 line to 16.2-fold in the OFV8 line.

MeJA-treated Plants The application of MeJA increased the production of volatile compounds overall (MANOVA, Wilks' $\lambda=0.030085$, $F=22.76$, *ndf*=17, *ddf*=12, $P<0.001$). Volatile production also varied among genetic lines (MANOVA, Wilks' $\lambda=0.000020$, $F=3.154$, *ndf*=119, *ddf*=88.2, $P<0.001$) and due to the interaction between MeJA treatment and genetic line (Wilks' $\lambda=0.000453$, $F=1.67$, *ndf*=119, *ddf*=88.2, $P<0.006$). The significant interaction shows that the responses to MeJA also differed among genetic lines. Application of MeJA increased the emission of (*Z*)-3-hexenyl acetate ($F_{1,\ 28}=10.84$, $P=0.003$), limonene ($F_{1,\ 28}=5.35$, $P=0.028$), (*E*)-β-ocimene ($F_{1,\ 28}=99.06$, $P<0.001$), linalool ($F_{1,\ 28}=8.06$, $P=0.008$), DMNT ($F_{1,\ 28}=4.54$, $P=0.042$), unknown sesquiterpene 2 ($F_{1,\ 28}=10.92$, $P=0.003$), (*E*)-β-caryophyllene ($F_{1,\ 28}=342.94$, $P<0.001$), α-bergamotene ($F_{1,\ 28}=19.1$, $P<0.001$), α-humulene ($F_{1,\ 28}=175.9$, $P<0.001$), unknown sesquiterpene 3 ($F_{1,\ 28}=11.1$, $P=0.002$), β-selinene ($F_{1,\ 28}=74.54$, $P<0.001$), and TMTT

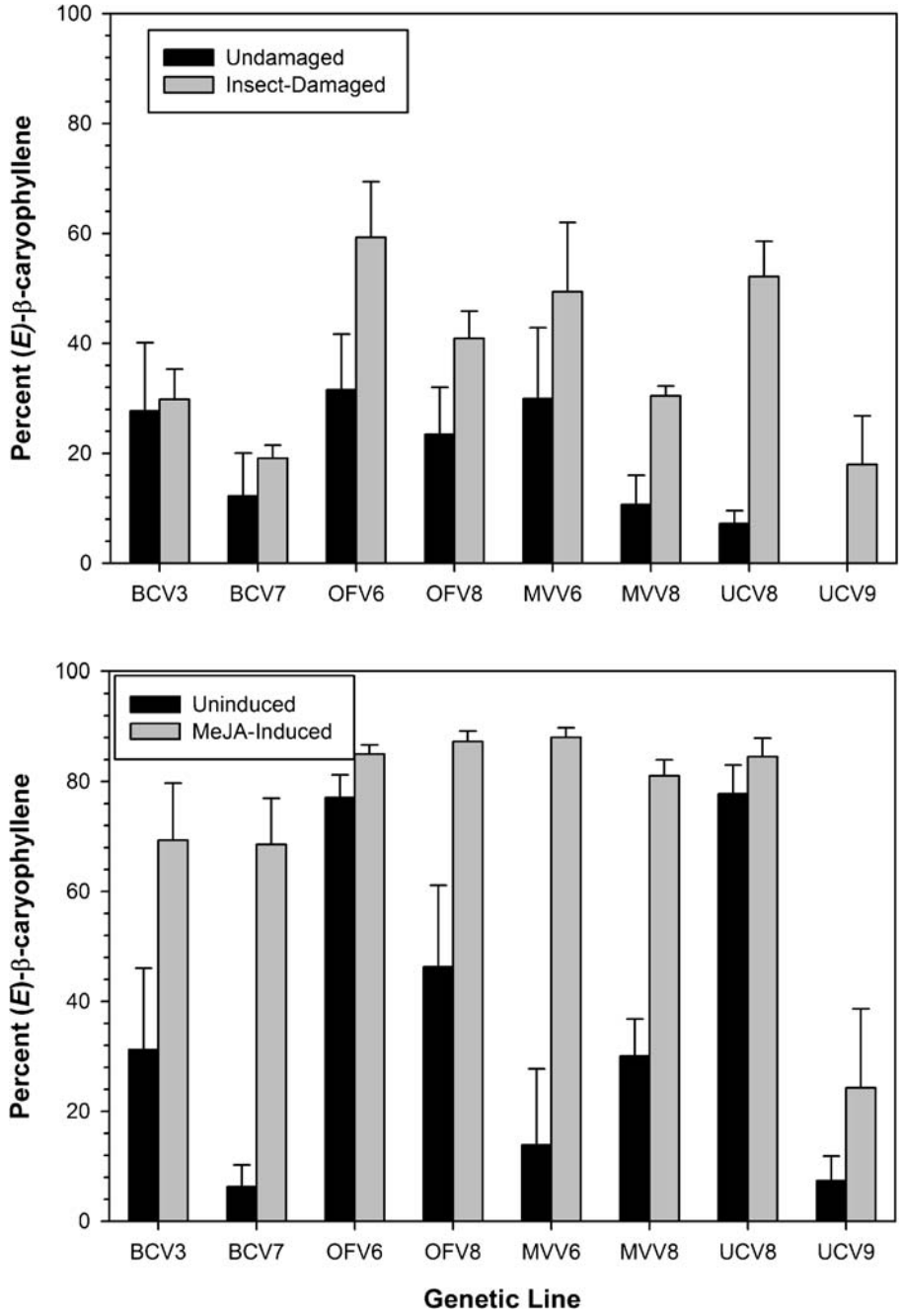


Fig. 1 Mean percent (+SE) of (*E*)-β-caryophyllene in the VOC blends of families from different genetic lines of *D. wrightii* pooled over trichome phenotype. Values are shown both before and after damage by *Lema daturaphila* (*upper plot*) or treatment with MeJA (*lower plot*)

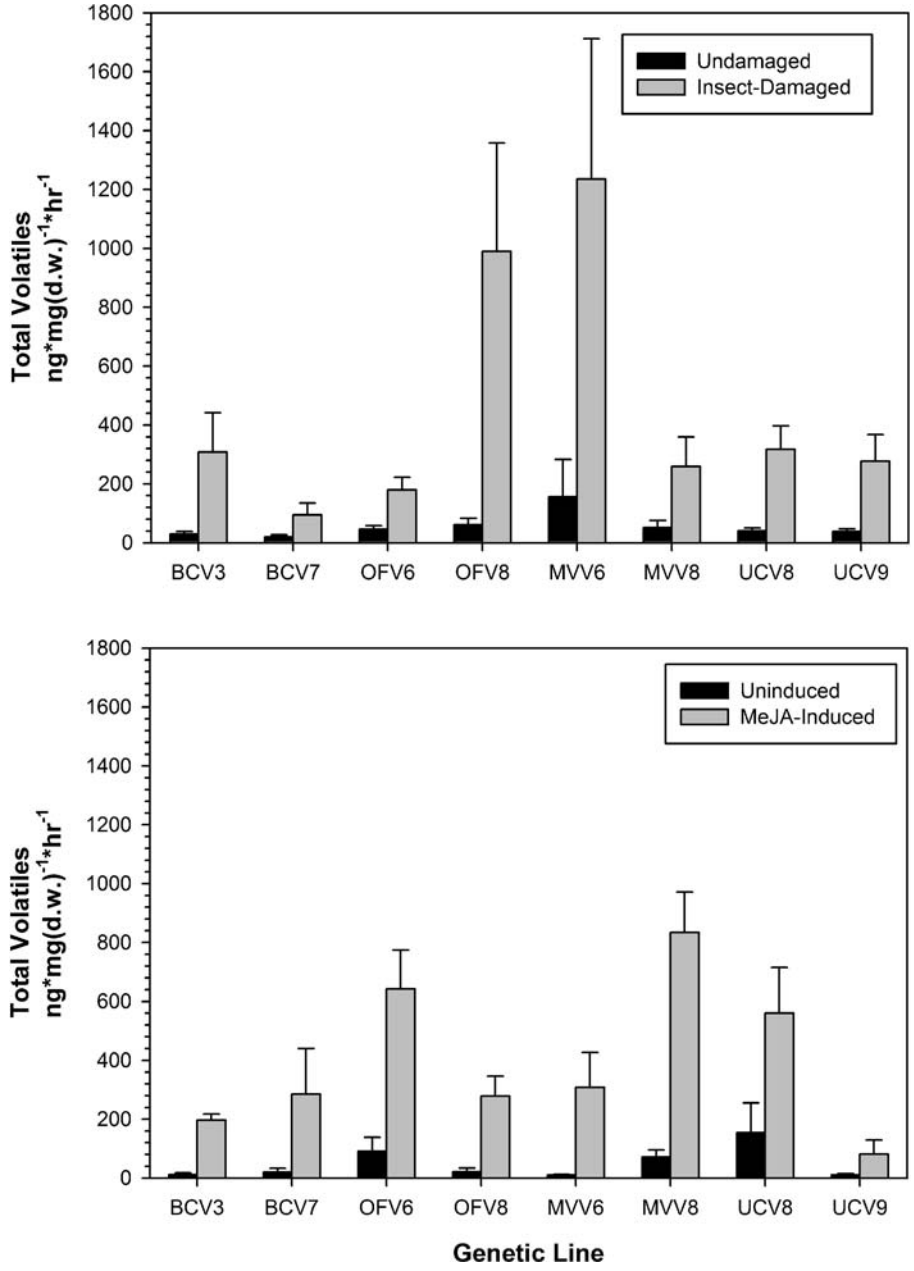


Fig. 2 Mean (+SE) total VOC production by different genetic lines of families from *D. wrightii* pooled over trichome type. Values are shown both before and after damage by *Lema daturaphila* (*upper plot*) or treatment with MeJA (*lower plot*)

($F_{1,\ 28}$=17.08, P<0.001, Table 1). The quantities of limonene ($F_{7,\ 28}$=12.73, P<0.001), (*E*)-β-ocimene ($F_{7,\ 28}$=4.39, P=0.002), decanal ($F_{7,\ 28}$=2.59, P=0.034), unknown sesquiterpene 1 ($F_{7,\ 28}$=10.10, P<0.001), unknown sesquiterpene 2 ($F_{7,\ 28}$=14.59, P< 0.001), (*E*)-β-caryophyllene ($F_{7,\ 28}$=6.58, P<0.001), α-bergamotene ($F_{7,\ 28}$=4.28, P<

0.003), α-humulene ($F_{7, 28}$=3.19, P=0.013), unknown sesquiterpene 3 ($F_{7, 28}$=10.85, P< 0.001), β-selinene ($F_{7, 28}$=3.64, P<0.007), nerolidol ($F_{7, 28}$=4.86, P=0.001), TMTT ($F_{7, 28}$= 4.71, P=0.001), and unknown sesquiterpene 4 ($F_{7, 28}$=3.12, P=0.014) also differed among genetic lines. Interactions between MeJA treatment and genetic line were statistically significant for the production of (*E*)-β-ocimene ($F_{7, 28}$=4.51, P=0.0018), (*E*)-β-caryophyllene ($F_{7, 28}$=5.33, P<0.001), and α-humulene ($F_{7, 28}$=2.79, P=0.025).

The percent (*E*)-β-caryophyllene differed due to MeJA treatment ($F_{1, 28}$=133.0, P< 0.001), genetic line ($F_{7, 28}$=8.31, P<0.001), and the interaction between genetic line and MeJA treatment ($F_{7, 28}$=9.12, P<0.001, Fig. 1). In the plants treated with MeJA, the percent of the total blend of volatiles that was (*E*)-β-caryophyllene ranged from 24.3% in the UCV9 line to 88% in the MVV6 line. Total volatile production differed significantly only between MeJA treatments ($F_{1, 28}$=255.2, P<0.001) and among genetic lines ($F_{7, 28}$= 4.41, P=0.002). Total VOC production increased from 3.6-fold in the UCV8 line to more than 32-fold in the MVV6 line (Fig. 2).

There was no significant variation due to trichome type nor due to any statistical interactions involving trichome type in the MANOVA analyses of either experiment (all $P \geq$ 0.13). Sibling plants differing in trichome type produce similar VOCs (Table 1).

Relationship between Parents and Offspring in (E)-β-Caryophyllene Production Although the same range of volatiles was collected from parents as offspring, for simplicity, only the relationship between parents and offspring for the most abundant volatile, (*E*)-β-caryophyllene, was analyzed and presented. The variation among genetic lines in their production of (*E*)-β-caryophyllene after treatment with MeJA in the backcross progeny was related to the variation in MeJA-induced parents (b=1.108, P=0.04, Fig. 3).

Discussion

Datura wrightii produces an array of terpenoids in response to damage by *Lema daturaphila*, its primary herbivore, or after treatment with MeJA. The amount of increase varied from 3.6- to over 32-fold among *D. wrightii* genotypes. In some cases, VOC production of some lines after insect damage or MeJA treatment was lower than the production of VOCs by other lines before damage or MeJA treatment. By far the most abundant compound was (*E*)-β-caryophyllene, and the proportional concentration of this compound in the total VOC blend after insect damage varied from 17–59% among genetic lines. Previously, PIs and PPO activity were shown to be inducible traits of *D. wrightii*, but the activities of these defensive proteins were independent of trichome phenotype. Similarly, in this study, inducible VOC production also was independent of trichome phenotype.

Variation in the production of VOCs has been studied primarily in cultivars of agricultural crops, including maize (Hoballah et al. 2004), cotton (Loughrin et al. 1995), rice (Lou et al. 2006), carrot (Nissinen et al. 2005), pear (Scutareanu et al. 2003), gerbera (Krips et al. 2001), and cruciferous crops (Bukovinszky et al. 2005). Variation in VOC production also is known in laboratory mutants of other species such as tomato (Thaler 2002) and lotus (Ozawa et al. 2000). In an analysis of 31 maize hybrid lines, for example, there was about 70-fold variation in the quantity of volatiles emitted and in the odor profile; in some cases, the quantities of odors emitted by undamaged plants of one line were higher than quantities emitted by damaged plants of other lines (Degen et al. 2004), as was the case in *D. wrightii*. The significance of such variation in VOC production among cultivars

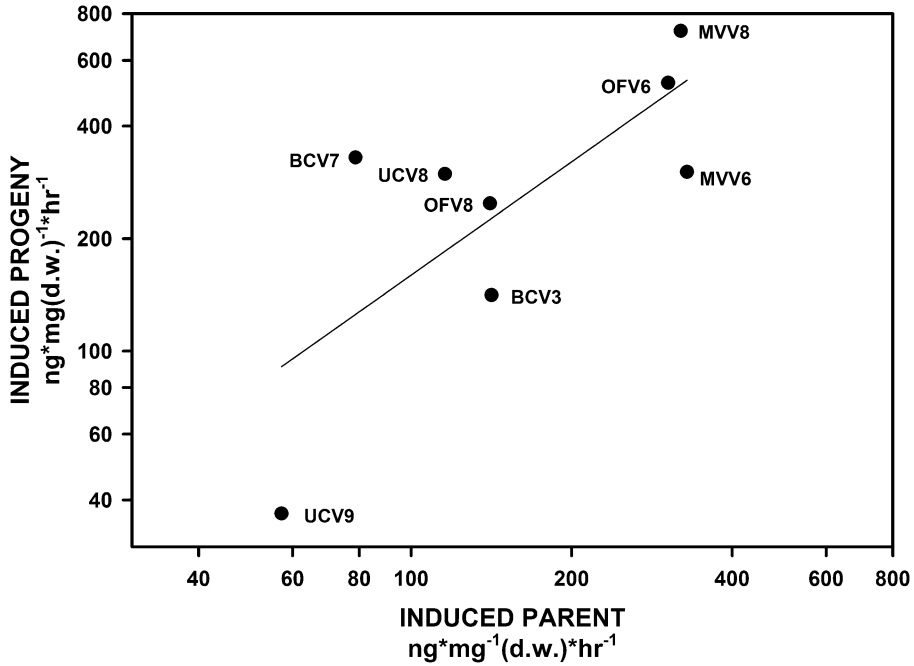


Fig. 3 Relationship between the production of (*E*)-β-caryophyllene by the original male parents of each genetic line and the family means of their third-generation backcross progeny. The regression line (slope= 1.108, intercept=−0.067) is statistically significant at $P=0.045$

is difficult to evaluate. Although some natural enemies may show differential attraction to the odors of particular cultivars in the laboratory (Krips et al. 2001; Hoballah et al. 2002; Bukovinszky et al. 2005), only rarely has it been shown that the more attractive cultivars actually benefit from enhanced biological control in the field (Lou et al. 2006).

Relatively little is known about the extent of genetic variation in VOC production of non-cultivated species, although such variation has been reported among five species and subspecies of teosinte, the ancestors of cultivated maize (Gouinguene et al. 2001). In that study, up to eightfold quantitative variation was noted among the five accessions, and qualitative differences, as indexed by the proportion of three major sesquiterpenes, were also observed. To date, the significance of such variation in VOC emissions among teosintes for suppressing the natural enemies of teosinte's native herbivores in its natural, ancestral environments remains unknown.

Another example of variation in VOC production in natural populations involves three populations of *Nicotiana attenuata* in the American southwest. Halitschke et al. (2000) compared two populations in Utah and found that they differed in their production of (*E*)-β-ocimene, linalool, and *cis*-α-bergamotene. Additionally, a population of *Nicotiana attenuata* in Arizona produced no induced VOCs (Glawe et al. 2003). Although the ecological significance of variation in VOC production between the two Utah populations remains unclear (Halitschke et al. 2000), the primary way in which the Arizona population may cope with absent or reduced induced defenses against its herbivores may be through increased competitive ability (van Dam and Baldwin 2003).

The MeJA experiment was performed to standardize the level of induction and, therefore, is the more accurate induction treatment to investigate potential differences in

induced VOC production among genetic lines. The significant, positive relationship between production of (*E*)-β-caryophyllene between male parents and backcross offspring suggests that the quantitative differences are inherited, although the experimental design was not appropriate to calculate formal estimates of genetic heritability for any plant population. Nevertheless, these data suggest that more detailed experiments to quantify the heritability of VOC quantities and blends within these populations could be worthwhile.

Although the MeJA and insect damage experiments were performed at different times and may not be directly comparable, it appears that feeding by *Lema daturaphila* and MeJA treatment have similar effects on the production of VOCs by *D. wrightii*. Both cause a substantial increase in the production of most compounds, although by far the greatest increases were in the production of (*E*)-β-caryophyllene. Damage by *Lema daturaphila* may cause relatively greater increases in the production of (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, DMNT, and β-selinene (Table 1). Differences in the volatile blends from MeJA-induced plant compared to insect-damaged plants may not be surprising if plants generally respond differently to insects than to MeJA. Such differences have been reported previously in other systems (Dicke et al. 1999; Degenhardt and Lincoln 2006; Rodriguez-Saona et al. 2006), and induction by insect damage generally results in a more complex blend of volatiles compared to induction with MeJA or JA alone, as was observed in the present study.

These results suggest numerous questions for further study on the evolution and maintenance of direct and indirect defenses against herbivores in *D. wrightii*. First, given the substantial quantitative and qualitative variation in VOC production of *D. wrightii*, do plants that produce either a greater quantity of VOCs, or a particular VOC blend, attract a greater number or diversity of natural enemies? If so, does such differential attraction result in differences in plant fitness? Is there additive genetic variation for total VOC production and odor profile? If so, and if differential attraction of natural enemies results in differential fitness, then is there natural selection on plants for the production of particular VOC blends? Alternatively, if plants producing different levels or blends of VOCs fail to differentially attract natural enemies, then the observed variation may merely reflect neutral variation, perhaps the result of stochastic processes such as genetic drift or founder effects within these relatively small plant populations (Hare and Elle 2004).

The answers to these questions will require detailed behavioral studies in both the laboratory and field on the natural enemies of *D. wrightii*'s herbivores. It seems unlikely that all of those species of natural enemy would respond similarly to variation in VOC production. In other systems, different herbivores induce the production of different VOC blends, and the different blends attract different, specialized parasitoids (e.g., De Moraes et al. 1998). Variation among herbivore-induced blends may matter less to generalist parasitoids (e.g., Yan and Wang 2006). Variation in VOC blends may be of even less relevance to *G. pallens*, which preys upon *Manduca* spp. and flea beetles (Kessler and Baldwin 2001, 2002), as well as *T. notatus* and *Lema daturaphila*, and is the predominant predator in Southern California populations of *D. wrightii* (Gassmann and Hare 2005). The fact that this species can be attracted to plants or sticky cards baited with single compounds (Kessler and Baldwin 2001; James 2002) suggests that only one or a few key components within a blend may be required to attract this important predator. The ecological importance of qualitative variation in the composition of VOCs emitted by *D. wrightii* genotypes after damage by *Lema daturaphila* may vary among different natural enemy species that attack *Lema daturaphila*.

The fact that sticky and velvety sibs produce similar VOC blends and quantities but differ in their suitability for foraging by resident natural enemies (Gassmann and Hare

2005) adds an interesting complication when coupled with prior observations that the frequency of plants with glandular trichomes varies widely among Southern and Central California populations (Hare and Elle 2001). If there is natural selection for VOC production, then the intensity of selection may vary among populations based upon the relative importance of direct defense against herbivores via glandular trichomes compared to indirect herbivore defenses mediated by VOC production. Selection for VOC production may be stronger in populations with a high frequency of plants that express nonglandular trichomes and weaker in populations with a high frequency of plants that express glandular trichomes. *D. wrightii* offers a rich system to investigate the ecological significance of variation in induced VOC production under natural conditions.

Acknowledgments I thank M. Haight and L. Christensen for assistance in the laboratory, J. G. Millar for assistance with the GC-MS analyses, and J. G. Millar and J. D. Allison for comments on a previous draft of the manuscript. This work was supported by the National Science Foundation under Grant no DEB 0414181 to J. D. Hare.

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antinutritional compounds, protein or nutritional constituents, leaf toughness, thorns, spines, or trichomes (Karban and Baldwin 1997). Following damage, plant nutritional quality may decrease or the concentration of secondary compounds, physical defenses, or volatile emissions may increase (Green and Ryan 1972; Karban and Baldwin 1997; De Moraes et al. 1998). In many cases, induced changes reduce subsequent herbivory and act as plant defenses (Carroll and Hoffman 1980; Karban and Baldwin 1997; Agrawal 2000). It is thought that induced defenses are less costly than constitutive ones because they are only activated after herbivory (Agrawal 2000; Strauss et al. 2002). Plant defense theory predicts a trade-off between induced and constitutive defenses (Koricheva et al. 2004).

Induced responses to herbivory have been demonstrated multiple times for leaf-chewing insect herbivores (Green and Ryan 1972; Carroll and Hoffman 1980; Malcolm and Zalucki 1996; Karban and Baldwin 1997), but less is known about the effects of phloem-feeding insect herbivores on induction. Because phloem-feeders do not remove leaf tissue per se, defenses induced by phloem feeders may differ from those induced by leaf chewers (Martel and Malcolm 2004). Induction by phloem feeders has been documented in a number of plant species (Gianoli and Niemeyer 1997; Gianoli 2002; Martel and Malcolm 2004; Sauge et al. 2006). In addition to variation among herbivore guilds, there is also variation among closely related plant species (Martel and Malcolm 2004; Cardoza et al. 2005). For example, previous work within *Asclepias* found evidence for cardenolide reduction and induction in high cardenolide species, but no change in cardenolide concentrations in species that lack constitutive defenses (Martel and Malcolm 2004). Similarly, interspecific variation in response to leaf-chewer herbivory has been documented in a number of genera including *Lupinus* and *Gossypium* (Thaler and Karban 1997; Vilarino et al. 2005).

In laboratory experiments, we measured changes in plant quality induced by varying densities of the milkweed–oleander aphid, *Aphis nerii*, on four species (*Asclepias incarnata*, *Asclepias syriaca*, *Asclepias tuberosa*, and *Asclepias viridis*). Specifically, we measured changes in defensive chemical concentrations (cardenolides), foliar nitrogen and carbon, and plant biomass. Plant defense theory predicts that species with high constitutive cardenolide concentrations will not exhibit induction, while species with low levels are more likely to (Karban and Baldwin 1997; Koricheva et al. 2004). We expected *A. tuberosa* (low constitutive cardenolides) to exhibit the highest degree of cardenolide induction, followed by *A. syriaca* (medium constitutive cardenolides), and we did not expect cardenolide induction in either *A. viridis* or *A. incarnata*.

Methods and Materials

Study System Asclepias is composed of predominately herbaceous perennials with a wide geographic distribution (Woodson 1954), and most produce a milky, cardenolide-laden latex when damaged (Malcolm 1991). Cardenolides, or cardiac glycosides, are a group of cardiac-active steroids that are bitter-tasting and toxic in high doses. They have many negative effects on herbivores, even in those species adapted to feed on them (Brower et al. 1984; Malcolm 1989, 1991; Malcolm and Brower 1989; Zalucki et al. 2001a). Their bitter taste combined with potential toxicity has implicated cardenolides in defensive use by plants against herbivores, parasites, and pathogens (Malcolm and Zalucki 1996; Agrawal and Malcolm 2002; Agrawal and Fishbein 2006).

There is much variability in cardenolide concentrations within and among milkweed individuals and species (Nelson et al. 1981; Malcolm 1991; Agrawal and Fishbein 2006)

and both qualitative and quantitative variation among *Asclepias* species (Malcolm et al. 1989; Malcolm 1991). Species also vary in carbon and nitrogen concentration, trichome density, specific leaf area, and water content (Agrawal 2004).

Aphis nerii Boyer de Fonscolombe (Hemiptera: Aphididae) is an aposematic, phloem-feeding specialist of milkweed, *Asclepias* spp., and oleander, *Nerium oleander* L. (Apocynaceae). It undergoes parthenogenetic reproduction for most of its life cycle, and sexual morphs are only produced under cold-day, long-night conditions (Takada and Miyazaki 1993).

Laboratory Experiments In August 2003, three *A. nerii* individuals were collected from geographically distinct locations (Atlanta, GA; Augusta, GA; and Gainesville, FL). Each is referred to as a separate clone. An aphid colony was initiated from one individual from each location, leading to three lab colonies. Separate colonies of these clones were kept at low densities on *A. syriaca* in the growth chamber.

Experiments were conducted in a temperature- and light-controlled walk-in growth chamber. Metal halogen grow lights on timers (16:8 hr, L–D) provided heat and light, and daytime and nighttime temperatures were 34±2.4 and 24±0.48°C, respectively. *Asclepias* seeds (Butterfly Encounters) were grown in Farfard 3B soil with 0.5 g of Osmocote time-release fertilizer (14:14:14, N–P–K) and watered as needed. Genotypes were unknown. Seedlings were grown in small pots (8 cm diameter); however, they were not root bound. Plants were randomly assigned to treatments and rotated daily to homogenize any environmental gradients within the growth chamber. Four species, *A. tuberosa*, *A. viridis*, *A. incarnata*, and *A. syriaca*, were chosen to represent a range of plant quality traits. They vary in foliar carbon and nitrogen concentrations, trichome density (Agrawal 2004), and cardenolide content (Malcolm et al. 1989; Agrawal 2004), as well as in their life history strategies. The rank order from lowest to highest cardenolide contents are *A. tuberosa* (negligible), *A. incarnata* (low), *A. syriaca* (moderate), and *A. viridis* (high) (M.D. Hunter, unpublished data). These four species were previously studied in a set of field experiments (Helms et al. 2004).

The identical experimental design was repeated on each of the four *Asclepias* species (*A. tuberosa*, June 2004; *A. viridis*, July 2004; *A. incarnata*, October 2004; and *A. syriaca*, November 2004). Because of time and space limitations, we could not examine all species concurrently. Thus, comparisons among plant species should be treated with caution. However, photoperiod, temperature, and other environmental conditions remained constant in the growth chamber over the course of all experiments. For each experiment, three aphid densities (1, 25, and 50 aphids per plant, and 0 aphids on controls) were established on 1-mo-old *Asclepias* seedlings that were grown in the chamber prior to the start of each experiment. Fifty aphids per seedling is approximately 250–500 aphids per gram DW plant tissue, which is at the high end of natural aphid densities in our system (Helms et al. 2004). Higher aphid densities, for example, 75 aphids per plant, kill *A. viridis* and *A. tuberosa* seedlings before the experiment concludes. At the start of the experiment, all seedlings were 4 wk old, approximately the same size (10–15 cm in height), and composed of a single stem bearing 6–10 leaves. Initially, five adult apterae aphids were placed on all plants, except controls, and left to reproduce overnight. Adult aphids were removed, and their offspring were thinned to the assigned density with a paintbrush. If necessary, offspring were moved from low- to high-density plants. This was repeated for all three clones, except for the experiment on *A. syriaca*, for which only the Emory and Florida clones were available. Each density by clone combination was replicated seven times, and each plant was caged (including controls). Cages were

constructed from 710-ml Ziploc containers and organza netting over wire frames. Initial cohorts were allowed to develop and reproduce. Once they started reproducing, all offspring were removed daily to keep densities constant. This continued until all aphids died (29±1 d), meaning that the size of each initial aphid population decreased over time.

For each plant species, seven individuals were harvested at the start of the experiment on the same day that the aphids were put on the plants (control A), and seven other plants served as controls throughout the experiment and received no aphids (control B). We chose these controls instead of measuring the same plants twice to avoid any induction caused by early leaf sampling on young plants.

Plants were harvested at the end of the experiment after all aphids had died. Each plant was cut at the stem base and rinsed with deionized water to remove honeydew. Plants were freeze-dried and weighed with a Mettler balance to determine above-ground plant biomass, then ground and homogenized. From each individual, a subsample (~2–3 mg) was analyzed for carbon and nitrogen and another subsample (~50 mg) for cardenolides (methods below). We analyzed the entire above-ground plant because aphids colonized both stems and leaves.

Plant Chemistry For carbon and nitrogen analysis, ground samples were weighed in tin capsules with a Mettler UMT2 microbalance (Mettler Toledo, Greifensee, Switzerland) and analyzed with a Carlo Erba NA 1500 CHN analyzer (Carlo Erba, Milan, Italy). Cardenolides were analyzed by using methods modified from Malcolm and Zalucki (1996). Briefly, ground samples were extracted with methanol and analyzed with high-performance liquid chromatography (HPLC). Peaks were detected by diode array at 218 nm, and absorbance spectra were recorded from 200 to 300 nm with digitoxin as the standard. Peaks with symmetrical absorption maxima between 217 and 222 nm were recorded as cardenolides (Malcolm and Zalucki 1996). Total cardenolide concentration was calculated as the sum of all individual cardenolide peaks.

Statistical Analyses Given that experiments were run sequentially, data from each plant species were analyzed separately. Total cardenolide concentration, plant biomass, and plant foliar nitrogen and carbon were compared among treatments by using two-way ANOVAs with aphid density and clone as main effects. Residuals were checked for normality, and data were transformed when necessary. *Asclepias viridis* biomass data were log transformed. Tukey's honest significant difference tests were conducted to compare among treatment means.

Neither aphid clone nor the interaction between aphid clone and aphid density was significant for any of the plant response variables measured for any of the plant species studied. Therefore, subsequent analyses were done by pooling the three aphid clones together within each density category. This allowed us to compare the three aphid density treatments with control B plants (no aphids). We reran the analyses for total cardenolide concentration, plant biomass, and foliar nitrogen and carbon by using a one-way ANOVA with aphid density (0, 1, 25, or 50 aphids per plant) as the main effect.

In addition to the response of total cardenolide concentration to variation in aphid density, we were interested in the response of individual cardenolide compounds. A MANOVA was used to analyze all individual peaks concurrently, and this was followed by separate univariate ANOVAs for individual peaks. Subsequently, *P* levels were adjusted downward because of multiple comparisons that used standard (nonsequential) Bonferroni methods (Sokal and Rohlf 1995).

Results

Milkweed species varied in their responses to aphid herbivory in general, and specifically to increases in aphid density. Because aphid clone had no effect on any of the response variables measured, we only report comparisons among the four density treatments (0, 1, 25, and 50 aphids per plant).

Aphid density influenced total cardenolide concentration in only one species. As density increased, total cardenolide concentration in *A. viridis* decreased (Fig. 1a $F_{3,62}$=5.86, P=0.001). There was a 43% reduction in cardenolides between the low- and high-density treatments. However, there was no difference between total cardenolide concentration of controls and *A. viridis* plants that experienced aphid herbivory. Eighteen individual cardenolide compounds were detected by HPLC in *A. viridis* (MANOVA Wilk's lambda $F_{54,135}$=1.83, P=0.003). However, only three of these (identified as peaks 17, 12, and 2) were influenced by aphid density (Fig. 1b: peak 17 $F_{3,62}$=9.54, P<0.001; peak 12 $F_{3,62}$=5.21, P=0.003; peak 2 $F_{3,62}$=5.56, P=0.002) with concentrations generally declining at the highest aphid densities.

In *A. syriaca*, overall cardenolide levels were more than an order of magnitude lower than those in *A. viridis* (average *A. syriaca* cardenolide concentration 0.22±0.04 mg/g). There was no effect of aphid density on total cardenolide concentration (data not shown, $F_{3,43}$=0.15, P=0.927), nor on any of the four individual cardenolide compounds detected (MANOVA Wilk's lambda $F_{12,106}$=0.82, P=0.63; individual ANOVA were all nonsignificant, data not shown). No cardenolides were detected in *A. tuberosa*, and cardenolides were detected in only one *A. incarnata* individual (that received 50 aphids per plant).

The effect of aphid density on plant growth (measured as above-ground biomass accumulation) varied among plant species (Fig. 2). As aphid density increased, plant biomass accumulation decreased by 58% on *A. viridis* ($F_{3,62}$=20.63, P<0.001). On *A. incarnata*, plants receiving the high-density treatment accumulated 24% less biomass than those receiving the low-density treatment ($F_{3,59}$=6.70, P=0.001). However, there was no difference in above-ground biomass accumulation between controls and treatment plants under Tukey's honestly significant difference (HSD) test. There was no effect of aphid

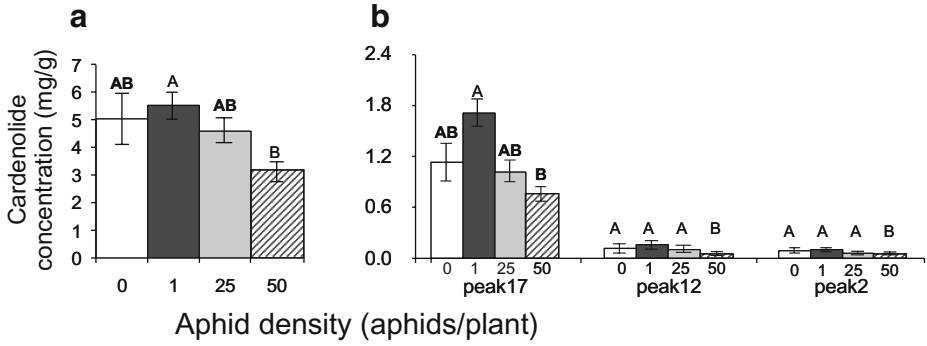


Fig. 1 The effect of increasing *A. nerii* density on **a** total cardenolide concentration and **b** individual cardenolide compounds in *A. viridis*. *White bars* are controls (0 aphids per plant), *dark gray bars* are the low density treatments (1 aphid per plant), *light gray bars* are 25 aphids per plant, and *striped bars* are 50 aphids per plant. There were no significant differences among the three aphid clones, so values were pooled in each density category. Values are means (N=7 for control, N=21 for all other density treatments) ±1 standard error. Different *letters* above *bars* represent significant differences among treatment means

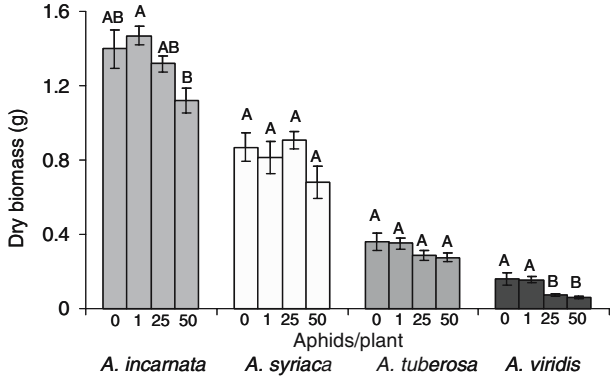


Fig. 2 The effect of increasing *A. nerii* density on final above-ground dry biomass of four *Asclepias* species: *A. incarnata*, *A. syriaca*, *A. tuberosa*, and *A. viridis*. Data from three aphid clones were pooled because there was no effect of clone on biomass accumulation. *Bars* are means of 21 plants (14 for *A. syriaca*) ±1 standard error except for controls (0 aphids per plant), which are means of 7 plants. Different *letters* above *bars* represent significant differences among treatment means within each plant species

herbivory on biomass accumulation of *A. syriaca* ($F_{3,43}$=1.60, P=0.203) or *A. tuberosa* ($F_{3,64}$=1.83, P=0.151).

Asclepias viridis individuals that received the highest aphid density treatment had 15% less foliar nitrogen than control individuals (Fig. 3: $F_{3,65}$=3.49, P=0.021). On *A. incarnata*, there was a trend towards increased foliar nitrogen concentration as aphid density increased ($F_{3,63}$=3.30, P=0.026), but none of the treatments was significantly different from the control under Tukey's HSD test. Aphid herbivory did not influence plant foliar nitrogen in either *A. tuberosa* ($F_{3,62}$=0.57, P=0.636) or *A. syriaca* ($F_{3,44}$=1.56, P=0.212).

Asclepias incarnata individuals that received the high-aphid-density treatment had 3% less foliar carbon than both control plants and plants that only had a single aphid on them (Fig. 4: $F_{3,63}$=4.77, P=0.005). Aphid density did not influence foliar carbon concentrations in any of the other three plant species (*A. tuberosa* $F_{3,62}$=0.28, P=0.840; *A. viridis* $F_{3,65}$= 1.77, P=0.162; *A. syriaca* $F_{3,44}$=1.14, P=0.343).

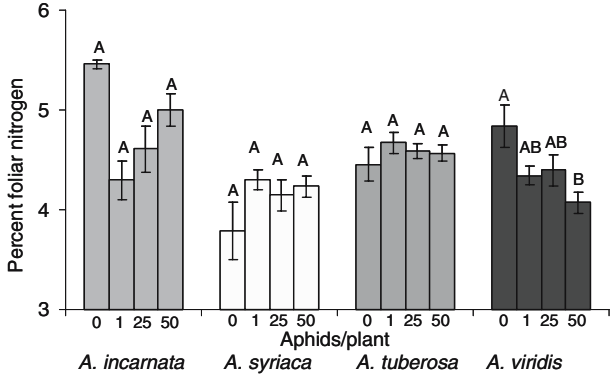


Fig. 3 The effect of variation in *A. nerii* density on foliar nitrogen of four *Asclepias* species: *A. incarnata*, *A. syriaca*, *A. tuberosa*, and *A. viridis*. Data from three aphid clones were pooled because there was no effect of clone on foliar nitrogen concentrations. *Bars* are means of 21 plants (14 for *A. syriaca*) ±1 standard error except for controls (0 aphids per plant), which are means of 7 plants. Different *letters* above *bars* represent significant differences among treatment means within each plant species

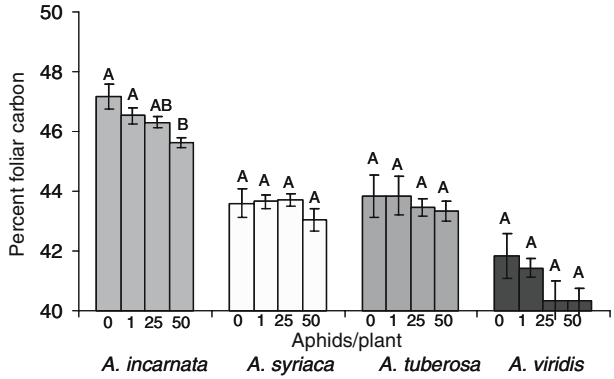


Fig. 4 The effect of variation in *A. nerii* density on foliar carbon of four *Asclepias* species: *A. incarnata*, *A. syriaca*, *A. tuberosa*, and *A. viridis*. Data from three aphid clones were pooled because there was no effect of clone on foliar carbon concentrations. *Bars* are means of 21 plants (14 for *A. syriaca*) ±1 standard error except for controls (0 aphids per plant), which are means of 7 plants. Different *letters* above *bars* represent significant differences among treatment means within each plant species

Table 1 shows plant quality data for the 1-mo-old (control A) seedlings that were harvested at the start of the experiment (before aphids were added), and for control B seedlings that were harvested at the end of the experiment. Interestingly, 4-wk-old *A. incarnata* seedlings had cardenolides present in low concentrations, while older plants expressed no cardenolides. Additionally, cardenolide concentrations increased in *A. viridis* but decreased in *A. syriaca* as plants aged. Foliar nitrogen concentrations decreased as *A. syriaca* and *A. tuberosa* seedlings aged, but foliar carbon concentrations stayed constant between young and old seedlings for all four plant species.

Discussion

There was significant variation among the *Asclepias* species in their responses to aphid herbivory and variation in aphid density. We found no evidence for a trade-off between constitutive and induced cardenolide defenses. None of the species studied exhibited cardenolide induction. *Asclepias viridis* was the only species to show an aphid-induced change in cardenolide concentration, which declined with increasing density (Fig. 1).

Table 1 Biomass, total cardenolide concentration, percent foliar nitrogen, and percent foliar carbon for control A seedlings (collected at the start of the experiment) and control B seedlings (collected at the end of the experiment) of four *Asclepias* species

	Biomass (g)[a]		Total Cardenolide Concentration (mg/g)[a]		Foliar Nitrogen (%)[a]		Foliar Carbon (%)[a]	
	A	B	A	B	A	B	A	B
A. incarnata	0.12±0.02	1.40±0.10	0.23±0.03	0	5.97±0.32	5.45±0.04	43.28±0.27	47.17±0.43
A. syriaca	0.12±0.22	0.87±0.78	0.50±0.23	0.20±0.06	6.16±0.16	3.78±0.28	44.24±0.21	43.60±0.50
A. tuberosa	0.05±0.01	0.36±0.05	0	0	5.54±0.20	4.46±0.17	43.58±0.45	43.83±0.70
A. viridis	0.02±0.01	0.16±0.01	2.98±0.56	5.02±0.89	4.86±0.22	4.84±0.21	40.22±0.55	41.83±0.74

[a] Values are means of seven plants ±1 standard error

Similarly, *A. viridis* foliar nitrogen concentrations declined with increasing density (Fig. 3). Both *A. viridis* and *A. incarnata* exhibited a decrease in final plant biomass with increasing density (Fig. 2). In contrast, aphids had no effect on the growth, nutritional quality, or defensive chemistry of either *A. syriaca* or *A. tuberosa*.

Previous research found that high *A. nerii* density induced cardenolide expression in *A. curassavica* but not in *A. incarnata* (Martel and Malcolm 2004). Martel and Malcolm (2004) found essentially no constitutive or inducible cardenolide defenses in *A. incarnata*, and we found no evidence for cardenolide induction in either of the low cardenolide species examined here (*A. incarnata* and *A. tuberosa*). These species may be defended by other compounds not measured here, such as pregnane glycosides (Warashina and Noro 2000).

The results of Martel and Malcolm (2004) for *A. curassavica* make an interesting comparison with our results for *A. viridis*, because both are high-cardenolide plant species. In *A. curassavica*, cardenolide concentrations decrease at low aphid densities, and then increase as density increases (Martel and Malcolm 2004). We observed no similar increase in cardenolide concentrations at high aphid density on *A. viridis* (Fig. 1a). Our plants were all young seedlings, and Martel and Malcolm (2004) used older plants. It is possible that aphid cardenolide induction or suppression varies with plant age, as has been shown for other plant species (Boege and Marquis 2005). It is also possible that there is much variation among *Asclepias* species in responses to aphid herbivory, such that *A. viridis* and *A. curassavica* simply respond differently to aphid attack.

We observed reduced growth of *A. viridis* and *A. incarnata* at high aphid density (Fig. 2). Because these experiments were run on small, young seedlings, we cannot make any generalizations about the impact of aphid herbivory on larger plants. In addition to causing a decrease in biomass accumulation, high aphid density led to a small decrease in *A. incarnata* foliar carbon concentrations (Fig. 4). While these results were statistically significant, we doubt that a 3% reduction in foliar carbon concentration is ecologically relevant for a plant like *A. incarnata* that typically grows in full sun.

Individual cardenolides differ in both their polarity and influence on herbivores (Malcolm and Zalucki 1996; Zalucki et al. 2001a, b), so it is not surprising that individual compounds respond differently to aphid attack. In *A. viridis*, only a subset (3 of 17) of cardenolide compounds declined significantly under aphid herbivory (Fig. 1b). An examination of the response of individual compounds will likely provide greater insight into plant responses to herbivory than measurements of total concentrations.

Mechanical damage to *A. syriaca* induces cardenolide expression after 24 hr, but then cardenolide concentrations drop to constitutive levels after 148 hr (~6 d) (Malcolm and Zalucki 1996). We collected plants approximately 29 d after each experiment started. If there was rapid cardenolide induction and a subsequent decay, we would have been unable to detect it. *Asclepias syriaca* may induce cardenolides in response to a simulated leaf-chewer, but not to phloem-feeder, damage.

Aphis nerii is a specialist of milkweed and oleander that can sequester cardenolides for defense against natural enemies (Malcolm 1989, 1991; Agrawal and Malcolm 2002). Some researchers have documented no difference in population growth between aphids feedings on high- and low-cardenolide plants (Groeters 1993; Martel and Malcolm 2004), while others have shown a negative impact of cardenolides on aphid maximum population growth (Agrawal 2004). Another milkweed specialist, the monarch butterfly, is negatively impacted by high levels of cardenolides (Zalucki et al. 2001a), so specialist herbivores are not necessarily immune from the defenses of the plants that they specialize on.

Assuming that the benefit to a specialist herbivore of sequestering cardenolides outweighs the negative impact of ingesting cardenolides, then from the plant's perspective,

cardenolide induction in response to herbivory by a specialist herbivore would be counterproductive. Increased cardenolide levels could actually benefit the herbivore and harm the plant because higher cardenolides would allow the specialist herbivore to be better protected from natural enemies. This is known as the lethal plant paradox (Malcolm and Zalucki 1996; Fordyce 2001). By not inducing cardenolides under aphid attack, all four *Asclepias* species avoid benefiting the cardenolide-sequestering herbivore at their own expense. Additionally, by reducing cardenolide concentration, *A. viridis* may reduce the cardenolide concentrations sequestered by *A. nerii* and thus lower the aphid's defense against natural enemies. Future work will compare sequestration of cardenolides under different aphid densities and examine variation in aphid predation and parasitism rates.

We have previously reported the results of aphid population growth on these plant species (Zehnder and Hunter 2007) and it is interesting to relate those dynamics to the plant quality traits measured here. *Aphis nerii* exhibits density-dependent survival, fecundity, and alate production on all four *Asclepias* species. However, density-dependent responses are strongest on *A. viridis* and weakest on *A. incarnata*. *Asclepias viridis* has the highest cardenolide concentrations of all the species we examined, and it also has the lowest biomass after 7 wk of growth. Our current results suggest that the density-dependent reductions in *A. viridis* cardenolide and nitrogen concentrations may be related to the density-dependent vital rates exhibited by *A. nerii*. However, changes in cardenolide, nitrogen, and carbon concentrations do not seem to be responsible for the density-dependent response of *A. nerii* on *A. incarnata*, *A. tuberosa*, or *A. syriaca*.

In summary, we found that changes in *A. nerii* density led to changes in plant chemistry and biomass, although not as we had predicted. *Asclepias* species with low constitutive levels of cardenolides did not exhibit higher levels of induced defenses than species with high constitutive defenses. In no case did increased aphid density lead to increased cardenolide expression. As aphid density increased, *A. viridis* cardenolide concentrations, biomass, and foliar nitrogen decreased. For *A. incarnata*, increased aphid density led to a decrease in plant biomass. Aphid density did not affect any measured trait of either *A. tuberosa* or *A. syriaca*. Our results highlight that congeneric plant species may respond differently to the same levels of herbivore damage.

Acknowledgements We thank J. Castings, P. Doty, M. Fleming, T. Maddox, and S. Scott for laboratory and field assistance. We thank B. Ball, W. Duncan, K. Wickings, and four anonymous reviewers for helpful suggestions concerning this manuscript.

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Myrmicinae) ants obligatorily colonize *Macaranga* and harvest its pearly secreta or food bodies as their main food source. The ants benefit the host plants by patrolling to exclude herbivores (Fiala et al. 1989; Fiala and Maschwitz 1990, 1992a, b). In obligate *Macaranga–Crematogaster* myrmecophytisms, neither party can survive in the absence of the other because they are highly specialized and dependent upon each other (Fiala and Maschwitz 1990; Fiala et al. 1999).

In contrast with other myrmecophytes from the American and African tropics, *Macaranga* is unique in having highly species-specific relationships with ants: one *Macaranga* species harbors one ant species principally, and one ant species interacts with a narrow range of *Macaranga* species (Fiala et al. 1999). Phylogenetic studies have noted the possibility of cospeciation in *Macaranga–Crematogaster* interactions (Itino et al. 2001; Quek et al. 2004), although partnerships of myrmecophytisms in other regions are less specific and appear to be determined by habitat segregation and colonization types, rather than cospeciation (Ward 1991; Davidson and McKey 1993; Yu and Davidson 1997). Moreover, *Macaranga* is ecologically unique among myrmecophytes in that multiple species of obligate myrmecophytes commonly coexist in similar microenvironments on a small spatial scale (Davies et al. 1998; Itioka et al. 2000); nevertheless, the species specificity is maintained (Itino et al. 2001).

The key factor that regulates specific partnerships is the wide variety of *Macaranga* defense strategies. There is interspecific variation in antiherbivore defense by symbiont ants, and ant aggressiveness differs among sympatric *Macaranga* species (Itioka et al. 2000). In addition, *Macaranga* species have different nonant defenses, i.e., physical and chemical defenses that complement ant defenses (Nomura et al. 2000). This suggests that different *Macaranga* species, including obligate myrmecophytes, have different strategies in terms of the balance between ant and nonant defenses. In contrast, the species complex of herbivores that feed on *Macaranga* and predators that feed on the symbiont ants also differ among *Macaranga* species (Itino and Itioka 2001). This is likely to be addressed by differences in defense strategies among *Macaranga* species. For example, *Macaranga* that tend to be attacked by specialist gall-forming flies are well-defended—both chemically and physically, whereas other *Macaranga* species that harbor the most aggressive ants have soft stems and leaves and suffer high levels of damage from birds that forage on the symbiont ants (Nomura et al. 2000; Itino and Itioka 2001). Each ant species is predicted to show its own specific aggressive behavior toward specific enemies in the mutualistic system between a *Macaranga* species and its symbiont ant.

When the host tree is injured, workers of the symbiont ant species immediately move from their nests inside the hollow stems to the surface of the plant and show aggressive behavior (Itioka et al. 2000). In addition, as in the *Cecropia* myrmecophytism (Agrawal 1998), aggressive behavior of symbiont ant workers is induced not only by damage to the host plant, but also by damage to neighboring myrmecophytic trees. This indicates that ant workers can recognize damaged areas, and that cues are released from the damaged host plant that stimulate aggressive behavior by the ants. Moreover, if ant workers inhabiting adjacent undamaged plants respond to damage-induced cues, the cues may provide useful information that enables the ants to recruit promptly for defense against a possible herbivore attack. Thus, in *Macaranga* myrmecophytisms, ant aggressive behavior would be induced not only by damage to the host tree, but also by damage to other *Macaranga* trees. Therefore, we examined whether damaged *Macaranga* leaves induce aggressive behavior in ant workers that inhabit a neighboring tree.

If ants respond equally to the damage of adjacent *Macaranga* trees regardless of tree species, ant defense induced by damage to adjacent trees would not be very effective because the aggressive ant behavior is considered to be enemy-specific. Unnecessary

frequently induced aggressive ant behavior may impose excessive costs on the ant colony, and subsequently, require the host tree to produce greater food rewards. If so, the cost–benefit balance of ant defense would be disrupted. Therefore, if symbiont ants on *Macaranga* myrmecophytes can distinguish between cues from conspecific and heterospecific host trees, they may avoid costly and ineffective responses to cues from adjacent *Macaranga* trees. Thus, we examined whether symbiont ant workers on *Macaranga* myrmecophytes show differential responses and aggressive behavior to artificial damage on adjacent *Macaranga* trees depending on tree species.

One possible cue that induces aggressive behavior in symbiont ants is volatile chemicals emitted from the injured area of the host plant. Many plants emit volatile compounds as an inducible response to herbivore damage that attract natural enemies of herbivores (Vet and Dicke 1992; Paré and Tumlinson 1999). Thus, we examined whether leaf volatiles emitted by injured *Macaranga* are species-specific, and therefore, may be used as chemical cues to induce ant defense.

Methods and Materials

Study site and materials The study site was located in a mixed dipterocarp forest in Lambir Hills National Park, Miri, Sarawak, Malaysia (4°2′ N, 113°50′ E; 60 m above sea level). The details of the study site were described by Yumoto and Nakashizuka (2005). More than ten myrmecophytic species of *Macaranga* occur at the site. We investigated four *Macaranga* species: *M. bancana* Miq., *M. beccariana* Merr., *M. trachyphylla* Airy Shaw, and *M. winkleri* Pax. & Hoffm. These are all obligate myrmecophytes inhabited by species-specific *Crematogaster* ants. The *Crematogaster* species inhabiting the four *Macaranga* species were classified morphologically and by mtDNA sequences (Itino et al. 2001), although the species have not been described. All experimental *Macaranga* trees were <2 m in height and were occupied by mature ant colonies. Field experiments and leaf volatile compound collections were conducted in August, September, and November 2004.

Assessment of ant defensive aggression To investigate whether the intensity of defensive attacks by resident ant workers stimulated by volatiles from injured leaves is specific to the host *Macaranga* species, a field assessment of ant aggressive behavior was conducted in at least ten randomly chosen trees of each of the four *Macaranga* species. The second leaf from the top of a different *Macaranga* tree was collected. A piece (1 cm^2) was cut from the leaf and held close to the second node of an experimental *Macaranga* tree by using forceps and without moving the tree. Leaf pieces were cut and used within 5 min of leaf collection. The numbers of ant workers that aggregated on the second node (recruits), climbed on the forceps or the experimenter's hand (climbers), and/or bit the experimenter's hand (biters) during a 90-sec period were recorded. For ants inhabiting the four *Macaranga* species, eight host leaf pieces were offered separately. For ants inhabiting *M. bancana*, a total of 20 nonhost leaf pieces (8 of *M. beccariana*, 8 of *M. trachyphylla*, and 4 of *M. winkleri*) were offered. For ants inhabiting *M. beccariana*, a total of 20 nonhost leaf pieces (8 of *M. bancana*, 8 of *M. trachyphylla*, and 4 of *M. winkleri*) were offered. For ants inhabiting *M. trachyphylla*, a total of 20 nonhost leaf pieces (7 of *M. bancana*, 8 of *M. beccariana*, and 5 of *M. winkleri*) were offered. For ants inhabiting *M. winkleri*, a total of 12 nonhost leaf pieces (4 of *M. bancana*, 4 of *M. beccariana*, and 4 of *M. trachyphylla*) were offered. When ants in a single experimental tree were examined more than once, we carried out experiments at intervals of at least 2 d, and leaf pieces were offered in randomized order to

eliminate influence by previous experiments. Forceps were rinsed with *n*-hexane between experiments to remove previous plant chemicals.

For each type of behavior within an ant species (represented by their host species), we compared the numbers of ants that responded among the *Macaranga* species by using Kruskal–Wallis tests, and compared the numbers of ants that responded between conspecific host leaf pieces and nonhost leaf species (pooled data) by using Mann–Whitney *U*-tests. For each type of behavior, Kruskal–Wallis tests were used for comparisons of the numbers of ants that responded among the ant species, and Steel–Dwass tests for multiple comparisons.

Chemical analysis of volatile leaf compound Volatile compounds emitted from injured young leaves were obtained by using headspace adsorption to examine differences in the chemical characteristics of the volatile compounds. The first three or four leaves were collected from the top of five randomly chosen trees per species for *M. bancana*, *M. beccariana*, and *M. winkleri*, and six randomly chosen trees of *M. trachyphylla*. The leaves were shredded into approximately 2-cm squares. We recorded the fresh weight of each set of leaves and then placed them into sampling bottles. The inlet of sampling bottles was connected via Tygon tubing to a purge bottle that contained activated charcoal and an air pump. The air was purified by the activated charcoal and pumped into the sampling bottle. Volatiles in the sampling bottle were adsorbed onto 50 mg of TENAX-TA (80–100 mesh; GL Sciences, Tokyo, Japan) in a glass tube (inner diameter [ID]=3 mm; length=16 cm] that was connected to the outlet of the sampling bottle. The air flux was maintained at 150 ml min^{-1} for 150 min. Empty sampling bottles were used as control.

The volatiles trapped in the TENAX-TA were eluted with 1 ml of diethyl ether. Each eluate was condensed to about 5 μl under N_2 flow, dissolved in 200 μl of hexane that contained 200 ng of *n*-eicosane as an internal standard, and 2 μl of each sample were analyzed. Analyses were performed with a GC-17A gas chromatograph and a QP5050A mass spectrometer (Shimadzu, Kyoto, Japan) equipped with a DB-WAX glass capillary column (J & W Scientific, 0.25 mm ID, 30 m length, 0.25 μm film thickness). Helium was used as the carrier gas. The injector was operated in splitless mode for 0.75 min. Oven temperature was programmed as follows: 40°C for 1 min, raised at 3°C min^{-1} to 90°C, to 200°C at 10°C min^{-1}, and then to 240°C at 20°C min^{-1}. Detected peaks were identified by comparing them with mass spectra in the NIST21, NIST27, NIST107, and NIST147 databases. Identification of each compound was further confirmed by comparison of both mass spectrum and GC retention data with those of authentic standards whenever possible. Peaks that were also detected in controls and those with extremely low intensity from only one individual of a species were omitted. Relative peak intensity was calculated as the ratio of the peak area to the peak area of the internal standard. Relative amounts of compounds in a sample were determined as relative peak intensities equivalent to 10 g (fresh weight) of leaves. Based on the relative amounts of compounds, compositions among *Macaranga* species were compared by using two-way repeated-measures ANOVA. For each compound, we compared the relative amounts among *Macaranga* species with one-way ANOVA. Statistical analyses were performed with JMP version 6.0J (SAS institute).

Results

Ant aggressive behavior induced by injured leaf pieces The number of ant recruits differed among *Macaranga* species, except for ants inhabiting *M. winkleri* (Fig. 1). The number of

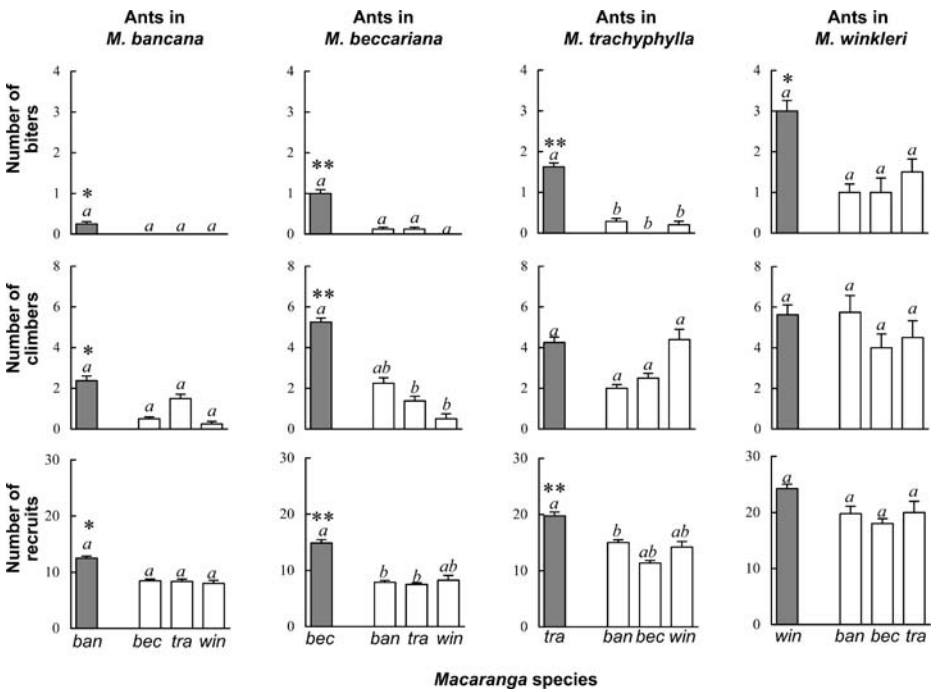


Fig. 1 Intensity of ant defensive aggression (mean number of ants±SE) for three types of aggressive behavior: recruiting, climbing, and biting against a leaf piece cut from another tree of *ban*: *M. bancana*, *bec*: *M. beccariana*, *tra*: *M. trachyphylla*, or *win*: *M. winkleri*. *Gray columns* mean ant responses when host conspecific leaf pieces were offered, and *open columns* mean when nonhost leaf pieces were offered. *Different alphabetical letters* indicate a significant difference in ant response among *Macaranga* species (Steel–Dwass test, $P<0.05$). *Asterisks* above the gray columns indicate significance in ant responses between the offers of host and nonhost leaf pieces (*$P<0.05$, **$P<0.01$, Mann–Whitney *U*-test, see detail in the text)

ants inhabiting the other three species that arrived at host leaf pieces was higher than the number that arrived at nonhost leaf pieces (Mann–Whitney *U*-test: adjusted $Z=-3.047$, -3.331, and -2.775; $P=0.002$, $P<0.001$, and $P=0.006$ for ants inhabiting *M. bancana*, *M. beccariana*, and *M. trachyphylla*, respectively). The number of biters differed among *Macaranga* species only for ants that inhabited *M. beccariana* and *M. trachyphylla* (Fig. 1), although the number of biters that arrived at host leaf pieces was higher than the number that arrived at nonhost leaf pieces in all *Macaranga* species (Mann–Whitney *U*-test: adjusted $Z=-2.279$, -3.470, -4.316, and -2.138; $P=0.023$, $P<0.\ 001$, $P<0.001$, and $P=0.032$ for ants inhabiting *M. bancana*, *M. beccariana*, *M. trachyphylla*, and *M. winkleri*, respectively). In contrast, the number of climbers differed among *Macaranga* species for ants inhabiting *M. bancana* and *M. beccariana* (Fig. 1), which arrived at host leaf pieces in higher numbers than at nonhost leaf pieces (Mann–Whitney *U*-test: adjusted $Z=-2.330$, -3.387; $P=0.020$, $P<0.001$ for ants that inhabited *M. bancana* and *M. beccariana*, respectively).

When host leaf pieces were offered, there were significant differences in the number of recruits and biters among the four *Macaranga* species (Kruskal–Wallis test: adjusted $H=14.790$, $P=0.020$ for recruits; $H=16.885$, $P<0.001$ for biters; $H=6.314$, $P=0.097$ for climbers). When nonhost leaf pieces were offered, there were differences in all three types

of behavior among the four *Macaranga* species (Kruskal–Wallis test: adjusted H=39.186, 19.363, and 26.049 for recruits, climbers, and biters, respectively; P<0.001 for all).

Chemical composition of leaf volatile compound We detected a total of 18 compounds in headspace collections from injured leaves of the 4 *Macaranga* species. Although the four species shared ten compounds, the remaining eight were restricted to one or two species. A total of 17 compounds were identified. The only remaining trace compound showed relatively low similarities with mass spectra in the libraries, and thus could not be identified (Table 1). Of the 18 compounds, the amounts of 9 'green leaf volatiles' (GLVs), including hexane alcohols, aldehydes, and esters, which were major and common components in all *Macaranga* species, did not differ significantly among species. In contrast, the amounts of three monoterpenoids differed significantly among the *Macaranga* species—two sesquiterpenoids were found only in or in the largest amount in *M. trachyphylla*, although the differences in amounts were not significant. Among the four remaining compounds, only nonanal was significant in amount, although all four compounds were restricted to one or two *Macaranga* species (Table 1). The composition of the 18 volatile compounds varied among the *Macaranga* species (two-way repeated-measures ANOVA, df=51, F=1.686, P=0.005).

Table 1 Average relative amounts of the volatile compounds detected from cut leaves of the four *Macaranga* species

Compounds	PTRI	*Macaranga* Species				One-way ANOVA	
		M. bancana	*M. beccariana*	*M. trachyphylla*	*M. winkleri*	*F*	*P*
Hexanal	1,080	0.11 (0.9)	0.04 (0.5)	0.07 (1.3)	0.11 (1.5)	0.612	0.62
(*Z*)-3-hexenal	1,141	0.21 (1.6)	0.09 (1.0)	0.29 (6.1)	0.03 (0.7)	1.611	0.23
β-Myrcene	1,161	–	0.15 (1.7)	0.08 (1.4)	–	4.314	0.02
Limonene	1,196	0.12 (1.2)	0.79 (9.1)	0.32(6.0)	0.33 (5.2)	3.332	0.05
(*E*)-2-hexenal	1,216	0.13 (1.0)	0.21 (2.3)	0.1 (2.0)	0.91 (9.9)	2.590	0.09
m/z :128*, 43, 41, 68, 67, 57, 53, 44, 86, 85	1,221	–	0.03 (0.3)	–	0.07 (1.1)	1.609	0.23
β-Ocimene	1,253	0.04 (0.8)	–	0.03 (1.0)	–	5.474	0.01
Hexyl acetate	1,273	0.07 (0.6)	0.16 (1.7)	0.07 (1.3)	0.59 (4.7)	1.893	0.17
(*Z*)-3-hexen-1-ol acetate	1,321	2.58 (19.4)	3.54 (37.4)	1.17 (22.5)	3.43 (21.0)	0.534	0.67
(*E*)-2-hexen-1-ol acetate	1,340	0.03 (0.3)	0.11 (1.2)	0.07 (1.4)	1.21 (8.0)	1.422	0.27
1-Hexanol	1,366	0.70 (5.4)	0.62 (7.4)	0.34 (5.2)	1.09 (8.0)	0.517	0.68
(*Z*)-3-hexen-1-ol	1,392	9.44 (65.5)	2.53 (29.1)	2.88 (44.9)	1.27 (18.0)	2.014	0.15
Nonanal	1,395	–	–	–	0.15 (4.8)	4.117	0.02
(*E*)-2-hexen-1-ol	1,415	0.23 (1.7)	0.68 (8.2)	0.10 (1.9)	4.11 (17.0)	1.102	0.38
1-Octen-3-ol	1,461	0.06 (0.5)	–	0.03 (0.4)	–	3.211	0.05
α-Copaene	1,489	–	–	0.04 (0.8)	–	3.060	0.06
β-Caryophyllene	1,594	0.11 (1.1)	–	0.16 (3.4)	–	2.857	0.07
Methyl salicylate	1,795	–	0.02 (0.2)	0.02 (0.6)	–	1.835	0.18

Average proportion (in %) are shown in parentheses.

The compounds are listed in order of programmed-temperature retention indices (PTRI) relative to *n*-alkanes on DB-WAX.

All compounds were identified by comparison with authentic standards, except for one compound for which mass fragments are listed with the inferred molecular ion first (*) followed by the base peak and other fragments in order of intensity.

Discussion

In field experiments, ant workers on *Macaranga* trees showed aggressive behavior when a cut leaf piece was placed onto the stem of the tree. We offered leaf pieces carefully so as not to disturb the trees or give physical signs to the resident ants. Moreover, the ants could not recognize the leaf pieces visually from inside the hollow stems. Nevertheless, resident ants immediately emerged from the internodes and aggregated around the offered leaf pieces. Therefore, it is unlikely that ants used visual or physical cues to recognize offered leaf pieces. Rather, it seems more likely that leaf volatile compounds were used as chemical cues for defensive attack because volatile compounds are remotely perceivable and only occur when leaves are wounded. The ants themselves may emit volatile compounds—e.g., alarm pheromones, in response to plant damage, which may recruit other defenders. However, in field experiments, we offered leaf pieces when no ants were present on the tree surface. The aggressive behavior was not observed before starting the experiments in the absence of cut leaf pieces, even when neighboring *Macaranga* trees were close. Thus, at least for initial defenders, aggressive behavior may be induced by volatile compounds emitted from leaves that are mechanically wounded. Ant aggressive behavior in response to volatiles from injured leaves of neighboring *Macaranga* trees would be adaptive as a preemptive defense against herbivores on adjacent *Macaranga* trees.

Ant aggressive behavior differed among the four *Macaranga* species. *M. winkleri* harbored the most aggressive ant species, followed by *M. trachyphylla* and *M. beccariana*; *M. bancana* was the least aggressive species. This pattern of ant aggressiveness is attributed to differences among *Macaranga* species in resource allocation strategies with respect to specific enemy fauna (Itioka 2005). For example, *M. winkleri*, which has a relatively high dependence on symbiont ants for its antiherbivore defense, may profit greatly by recruiting preemptive ant defenders. Ants were recruited on trees of all four *Macaranga* species, even when leaf pieces of nonhost species were offered. Thus, ant recruitment may be induced by volatile compounds common among the leaves of at least these four *Macaranga* species.

In addition, there were intraspecific differences in the intensity of aggressive behavior: resident ant workers showed higher aggressiveness in response to host leaf pieces than to nonhost leaf species. Biting was the most definitive defensive attack behavior, and the number of biters was higher when host leaf pieces were offered than when nonhost leaf pieces were offered in all four *Macaranga* species. This suggests that ant workers can distinguish among injured *Macaranga* species, thereby determining their level of aggressiveness.

Chemical analyses verified that the composition of volatiles emitted from injured leaves was specific to each *Macaranga* species, and that several minor compounds were specific to one or two *Macaranga* species. Of the ten compounds that were detected in all four *Macaranga* species, nine were common GLVs. GLVs consist of a blend of saturated and unsaturated hexane alcohols, aldehydes, and esters, and may provide useful information regarding plant wounds to symbiont ants because GLVs are emitted immediately in response to mechanical plant cell damage (Galliard 1978; Pichersky and Gershenzon 2002) and in predominant amounts from cut leaves (Table 1). A certain number of ant workers showed aggressive behavior in response to nonhost leaf pieces, especially aggregating and climbing behavior (Fig. 1), presumably as a response to GLVs. The other 9 of 18 compounds were detected from 1 or 2 *Macaranga* species only, or in different amounts from all 4 *Macaranga* species, although many of these compounds occur in a wide range of plants. These species-specific compounds may play a role in inducing higher symbiont ant aggressiveness, e.g., the higher number of defenders in the partner ant species.

The level of ant aggressive behavior in the field experiment may have been induced by chemicals emitted from the first defenders that recognized leaf pieces. For example, ants inhabiting *M. winkleri* were the most aggressive, regardless of *Macaranga* species, presumably because their defensive behaviors are strongly controlled by pheromone perception. However, in terms of biting behavior, ants inhabiting *M. winkleri* responded in larger numbers to host than to nonhost leaf pieces (pooled nonhost data). This difference in biting intensity behavior in ants inhabiting *M. winkleri* implies that they could distinguish among leaf species. Wounded *M. winkleri* leaves emitted nonanal, which was not detected in the other three species, as well as a large proportion of (*E*)-2-hexen-1-ol and a small proportion of (*Z*)-3-hexen-1-ol, in contrast to the other three species. Undamaged leaves of *Macaranga* do not emit these green leaf alcohols or nonanal (Jürgens et al. 2006). Thus, the composition of volatile compounds may provide information for ants to distinguish whether or not a wounded plant is a host. If ants distinguish among the blends of volatile compounds emitted by wounded *Macaranga* leaves, aggressiveness may also differ among leaf pieces of nonhost species; however, we did not detect patterns between volatile composition and ant aggressiveness. However, this is not surprising if each ant species is specialized to respond to a small number of host-specific key compounds, such as nonanal in the *M. winkleri* symbiosis. To determine which compounds induce ant behaviors, further electrophysiological studies are required. We conclude that ant workers on *Macaranga* species recognize plant volatiles, including GLVs, as signals that elicit their preliminary orientation and aggregation to the injured area of host plants. They likely then discriminate minor and specific compounds to elicit critical defensive responses such as biting.

This process is important for the maintenance of the species specificity of *Macaranga–Crematogaster* mutualisms. Host-plant selection by foundress ant queens based on chemical cues plays a role in maintaining species specificity (Inui et al. 2001). *Macaranga* seedlings have species-specific nonvolatile surface chemicals that are used by foundress queens to recognize their partner plant species. In addition, the species-specific glaucous surface structure of *Macaranga* stems is a structural barrier preventing colonization of nonpartner ant species (Federle et al. 1997) and may also play a role in the maintenance of species specificity. However, these chemical and structural factors do not completely enforce species specificity during the colonization process because some *Macaranga* seedlings are colonized at low frequencies by foundress queens of nonpartner ant species (Murase et al. 2002; Jürgens et al. 2006). Because species specificity is well maintained in obligate myrmecophytic *Macaranga* trees occupied by mature ant colonies, there must be mechanisms that work after colonization to exclude such 'errors' in the establishment of specific pairs. The efficiency of ant defenses may be one mechanism that reinforces species specificity. Because differences in the intensity of ant defenses have been recognized as differences in allocation to defense strategies among *Macaranga* species (Itioka 2005), *Macaranga* trees colonized by mismatched, less aggressive ants would not survive herbivory, and those colonized by mismatched, more aggressive ants would not produce sufficient rewards to maintain the resident colony. Thus, the specific response of resident ants to host damage would be important for plant survival of herbivory, and consequently, for the survival of the resident ants on the plants.

Overall, our results suggest that *Macaranga* leaf volatiles are important chemical cues used by resident ant workers in guarding their host tree. This response to chemical cues may be a mechanism that gives rise to well-adapted biotic ant defense in species-specific *Macaranga–Crematogaster* associations, and by extension, sympatric cospeciation between ants and plants.

Acknowledgements We thank Ms. Lucy Chong of the Forest Research Center, Sarawak Forestry Corporation Sdn. Bhd., Abang A. Hamid Karim of the Department of Agriculture, Sarawak, and Prof. Tohru Nakashizuka of the Research Institute for Humanity and Nature for providing the opportunity to carry out this study in Lambir Hills National Park, and Mrs. Tadahiro Ohkubo and Shimpei Nishino of Osaka Kyoiku University for helping with the field assessments. We also thank the Center for Ecological Research, Kyoto University where we carried out the chemical analyses. This work was financially supported by grants from the Japan Ministry of Education, Culture, Sports, Science and Technology, Grant-in-Aid for Young Scientists (B, 15770008) to Y. Inui.

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Introduction

Volatile organic compounds (VOCs) emitted by plant–herbivore interactions are of importance for host or prey location by parasitoids and predators of phytophagous insects (e.g., Vinson et al. 1987; Dicke et al. 1990a, b; Turlings et al. 1995; Karban and Baldwin 1997). More than 1,000 VOCs are involved in such interactions (D'Alessandro and Turlings 2006), and in a single plant–herbivore complex 30–50 VOCs are frequently detected by chromatographic analysis. Some entomophagous arthropods are able to discriminate between minor qualitative or quantitative differences among this vast number of compounds, and use these differences as reliable cues to find hosts or prey. For example, sprouts infested by *Plutella xylostella* emit 10 additional VOCs compared to uninfested sprouts that emit 49 compounds. These qualitative differences are perceived by the parasitoid wasp *Diadegma semiclausum* that prefer the odor of infested plants (Bukovinszky et al. 2005). Among the myriad of VOCs identified, some are common and emitted in many plant arthropod interactions, whereas others are highly specific to a particular interaction. It is generally admitted that in tritrophic interactions that involve a carnivorous species specialized toward a particular phytophagous insect, the VOCs used by foragers are highly specific (Vet and Dicke 1992; Steidle and Van Loon 2003). Nevertheless, there is growing evidence that some specialist insects also use general cues such as β-farnesene (Mumm and Hilker 2005), methyl salicylate (de Boer and Dicke 2004), or green leaf volatiles (Whitman and Eller 1992). To understand how individuals use the cues of their environment in a way that can lead to an optimized foraging strategy (Bernays 1996), it is crucial to unravel how predators and parasitoids are able to use more or less specific compounds as reliable foraging cues. However, most studies that examine the effects of VOCs on the behavior of parasitoids or predators have been carried out in laboratory conditions (e.g., Vinson et al. 1987; Dicke et al. 1990a; Turlings et al. 1995; Sabelis et al. 2001; de Boer and Dicke 2004). Field studies are scarce (but see De Moraes et al. 1998 or Kessler and Baldwin 2001), and direct evidence for the potential of synthetic VOCs as field attractants for beneficial insects has only recently been obtained (James 2003, 2005).

Delia radicum, the cabbage root fly, is an economically important pest of cabbage crops (Finch 1989). Females lay their eggs in small clusters in the immediate vicinity of stems of Brassicaceae plants. Larval development takes place inside the roots, where larvae dig galleries while feeding, leading to root decay when infestation is heavy. A large part of egg and larval mortality in *D. radicum* is caused by predation by several species of carabidae and staphylinidae (Wishart et al. 1956; Hugues 1959; Coaker and Williams 1963; Luff 1987; Wright et al. 1960; Mowat and Martin 1981).

In this study, we investigated VOCs used in the field by the main natural enemies of *D. radicum* that are naturally found in cole crops (Finch 1989): small carabid beetles belonging to the genus *Bembidion* and the rove beetles *Aleochara bilineata* and *A. bipustulata* (Coleoptera : Staphilinidae). In both cases, adults are predators of *D. radicum* eggs and larvae, but the staphilinid larvae also develop as parasitoids of *D. radicum* pupae (Fuldner 1960). The two Aleochara species have been suggested previously as potential biological control agents against *Delia antiqua*, a pest of Alliaceae plants (Tomlin et al. 1985; Finch 1989). Whereas carabid beetles are generalist predators, *A. bilineata* and *A. bipustulata*, because they are also parasitoids of *D. radicum*, are more specialized. However, despite their close morphology and biology, the two staphilinids differ in host ranges. Whereas *A. bilineata* is a specialist of phytophagous and saprophagous Anthomyiidae dipterans, *A. bipustulata* has a wider host range, parasitizing not only Anthomyiidae species but also coprophagous and necrophagous non-Anthomyiidae dipterans. This difference in specificity should translate into a different use of volatile cues.

Only a few studies have examined the role of VOCs in host-finding behavior of *D. radicum* predators. In laboratory conditions, by using a Y-tube olfactometer, Royer and Boivin (1999) showed that volatiles coming from host-infested plants, as well as odors from larval frass and hydrosoluble volatiles coming from the larval tegument, were attractive to *A. bilineata* adults. However, the volatiles involved were not precisely identified. Another study highlighted the role of onion in mixed-crop strategies to improve the presence of *D. radicum* natural enemies, *A. bilineata*, *A. bipustulata*, and carabid beetles (Uvah and Coaker 1984). Nevertheless, it was unclear whether the observed effects were caused by onion volatiles or by confounding effects like the presence of additional resources. Finally, two field studies have revealed the ability to increase *A. bipustulata* catches in brassica plots that contain mustard seed meal (Ahlström-Olsson and Jonasson 1992; Riley et al. 2007). Which compounds, among the 14 identified in the natural odor source, could be responsible for this attraction was not specified.

To unravel the foraging cues used by *A. bilineata*, *A. bipustulata* and carabid beetles, to locate *D. radicum*, we first analyzed in the laboratory the VOCs emitted by the rotten part of *Brassica napus* roots that were heavily infested by *D. radicum* larvae. Then, by using an original bioassay designed for ground-dwelling beetles, we analyzed in the field the attractive effect on *D. radicum* natural enemies of various concentrations of the main VOC found compared to the odor emitted by naturally infested roots.

Methods and Materials

Natural Odor Source Infested roots used for the chemical analysis of volatiles and for the bioassays were obtained from our *D. radicum* rearing facility at the University of Rennes (France). Infestations were carried out in a controlled room (21±1°C, 60% RH, 16L:8D photoperiod). To obtain heavily infested material, swede roots (*Brassica napus* var. napobrassica) were placed on a bed of oven-dried sand in a glass plate and then put in 1× 1×1 m Plexiglas™ cages that contained a few hundred *D. radicum* adults. After 3 d, the plates containing the roots harboring many eggs were removed and placed into an insect-proof empty cage. *D. radicum* larvae were left to develop in the roots for 3 wk before the material was used in the experiments. The high level of infestation chosen corresponds to what can be found in the field after the first emergence peak of the flies.

Identification of Major Compounds Emitted by Heavily Infested Roots Three grams of the rotten part of heavily infested roots were put into a 30-ml glass vial closed with aluminum foil. Five repetitions of samples taken from different roots were carried out. Volatile sampling was obtained by inserting a solid-phase micro-extraction (SPME) fiber (1 cm long, 100 μm polydimethylsiloxane coating, SUPELCO 57300-U) through the aluminum sheet into the free space of the vial, where it was maintained for 15 min. Qualitative analysis was carried out with a Perkin–Elmer GC AutoSystem gas chromatograph (GC) coupled to a Perkin–Elmer Turbomass quadrupole mass spectrometer (MS). The GC column was a SGE BP1 capillary column (length 25 m, inner diameter 0.32 mm, film thickness 0.5 μm). The SPME fiber was inserted into the GC injector for thermal desorption in splitless mode for 2 min, and the split flow was adjusted to 30 ml/min. The injector was maintained at 250°C. The GC oven remained at 50°C for 2 min, then raised to 300°C at 5°C/min; helium was used as a carrier gas at a constant flow rate of 1 ml/min; the transfer line to the MS was thermostatted at 280°C, and the ion source at 180°C. MS spectra were recorded from 30 to 500 amu per 0.6 sec in the electron ionization mode at an electron energy of 70 eV

and a filament emission of 200 μA. Identification of molecules was carried out by using the internal NIST library, our personal library, and was confirmed by using pure standards when possible.

Quantitative Measurement of Dimethyl Disulfide (DMDS) All volatiles were collected by using a static headspace method. Headspace consisted of a 1-l airtight closed glass jar. A hole in the middle top of the jar permitted us to insert a SPME fiber for volatile sampling and the pure molecule dilutions for calibration. To allow odor concentration into the headspace, this hole was filled up with patafix™. Odors were sampled during 10 min, with the SPME fiber (same reference as above) at room temperature (24±1°C).

Quantitative measurements were carried out with a Perkin–Elmer GC-FID AutoSystem gas chromatograph (GC) equipped with a flame ionization detector (FID). The GC column was a Perkin–Elmer PE-5ms (5% diphenyl dimethylpolysiloxane) capillary column, (length 20 m, inner diameter 0.18 mm, film thickness 0.18 μm). SPME injection and oven program were the same as described for the GC-MS used in the qualitative analysis.

For calibration, a constant quantity of 1 μl of solution that contained DMDS diluted in paraffin oil was inserted with a Pipetman™ through the hole of the jar, and deposited on a small piece of filter paper maintained inside the jar with Patafix™. As soon as the droplet was deposited, the hole was closed for 5 min, permitting the volatile to evaporate before SPME sampling. The net quantities of DMDS used for calibration were: 1, 0.5, 0.1, 0.05, 0.03, and 0.01 μl. For each of these volumes, the measurement was repeated three times with new prepared solutions. Calibration measurements were conducted over the whole period of chemical analysis. The results showed a strong correlation between the FID peak area and the volume of DMDS used (peak area=8×10E-07 μl DMDS; R^2=0.9726).

Quantity of DMDS Emitted by Heavily Infested Roots Infested roots were weighted and placed in the jar 1 hr before volatile sampling to allow odor concentration to build up. After this period, the quantity of DMDS emitted was measured as described above. Quantities emitted were expressed in nanoliters of DMDS produced per gram of fresh product per hour (nl g^{-1} hr^{-1}). Measures were repeated on five different roots.

Quantity of DMDS Emitted by Field Traps Two microliters of DMDS diluted in 18 μl of paraffin oil were deposited on a 1-cm^2 piece of filter paper inside the trap (see trap description below). Traps were opened in the same way as in the field bioassay and kept 1, 5, or 24 hr on a work surface under an extractor at room temperature (24±1°C). After this period, traps were placed into the 1-l jar and left to emit for 1 hr before SPME sampling. Measures were repeated on three different traps.

Field Bioassays Bioassays were conducted on a 20×40 m broccoli plot in the experimental field station of La Rimbaudais in Saint Meloir des Ondes (48.65°N, 1.9°W), Brittany, France, in the heart of an important zone of cabbage production. Broccoli plants (*Brassica oleracea Italica* var. monopoly) were 8 wk old at the time of the experiment. Experiments were carried out on June 28 and 29 and July 4, 2006 under sunny weather (mean temperatures of 18.5, 18.3, and 22.5°C).

Setup The setup consisted of a rectangular grid of 90 equally spaced mini-pitfall traps arranged over nine rows of culture with 10 traps per row (spacing between and among rows was 80 cm, Fig. 1). Accordingly, the position of each trap in the setup could be identified

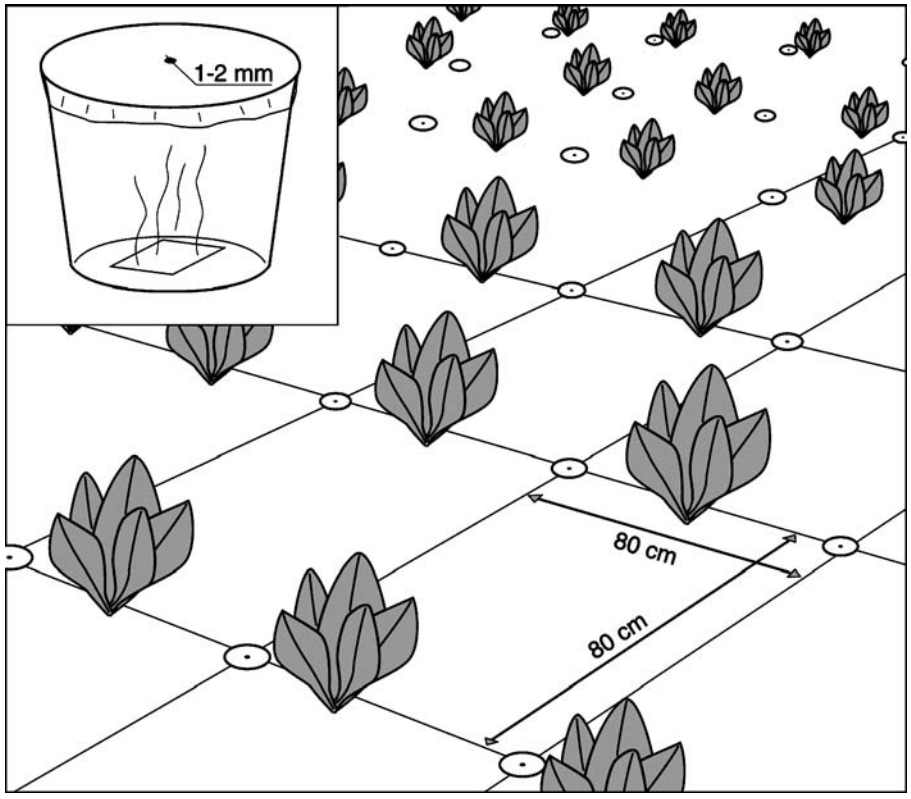


Fig. 1 Diagram showing the traps used to catch *Aleochara bilineata*, *A. bipustulata*, and carabid beetles of the genus *Bembidion* and their disposition in the field bioassays. A rectangular grid of 90 equally spaced traps was arranged over nine rows of broccoli plants with 10 traps per row (spacing between and among rows was 80 cm)

by its row number (1–9) and its column number (1–10). The location of the setup in the parcel was changed for each date the experiment was carried out.

Traps were made of 35-ml plastic cups (33 mm high, 40 mm upper diameter, P100 Solocup™). They were filled with odor sources in the field station's lab as follow: 1 g of the rotten part of an infested rutabaga root (R) or 20 μl of DMDS diluted in paraffin oil at the concentrations of 0% (C), 0.1% (D002), 1% (D02), 10% (D2), and 100% (D20), corresponding to a net quantity of 0, 0.02, 0.2, 2, and 20 μl of DMDS. As soon as an odor source was added, the cup was hermetically sealed with a piece of parafilm™. The 15 traps per treatment were randomly spatially distributed on the grid by using the internal function "sample" of R. Traps were placed in the field between 12:00 A.M. and 1:00 P.M. so that the upper part of the cup would be flush with the soil surface. After 1 hr, traps were carefully opened by piercing the piece of Parafilm™ in the middle with a precision forceps. Holes made this way were about 2 mm in diameter and permitted both odor diffusion and insect trapping. To limit variations caused by diel activity cycles, trapping was allowed for a 24-hr period without any perturbation in the field plot.

In parallel to the setup described above, a transect of 10 pitfalls (10 cm diameter) filled up with 50% alcohol and water, placed perpendicular to rows inside the parcel, was used to

monitor sex ratios of *A. bilineata* and *A. bipustulata* in the field. These pitfalls will be referred to as "control pitfalls" in the "Results" section.

Recovery of Trapped Insects Traps were brought to the lab for insect recovery. For each trap, we noted the treatment and position of the trap (row, column) as well as the number, species, and sex (determined with the presence of the male parameres, i.e., male genital apparatus) of *A. bilineata* and *A. bipustulata*, the number of other staphylinids, carabids, other coleopterans, dipterans, arachnids, and extra information such as dead individuals. All collected insects were grouped by date and treatment and conserved in alcohol for further investigation.

Statistical Analysis Statistical analyses were done with R software (R Development Core Team 2006). The numbers of trapped insects were analyzed with general linear model (GLM) statistic using the numbers of individuals caught in each trap. The distribution of the data was consistent with a Poisson distribution, and the linking function "log" was chosen for these GLMs. Factor effects were analyzed with the function "anova.glm" of R, using a chi-squared test, on a complete model that contained the following effects: treatment (i.e., the five concentrations of DMDS: C, D002, D02, D2, and D20, and R), date, treatment × date interaction, row, column. Numbers of individuals belonging to the same taxon trapped in different odors were compared with an analysis of contrast, using the function "esticon" of the package "doBy" (Højsgaard 2004).

The goodness-of-fit of each model was assessed by a graphical observation of the quantile randomized residuals (Dunn and Smyth 1996), plotted against the normal distribution quantiles (normal probability plot) by using the function "qqnorm" (Becker et al. 1988). Quantile randomized residuals were calculated using the function "qresiduals" from the R package "statmod" (Smyth 2005). The use of randomized quantile residuals instead of residuals *per se* was more appropriate because of: (1) the use of a Poisson family for the GLM fit, and (2) the fact that there were only a few possible values for the response (typically 0, 1, 2, 3) (Dunn and Smyth 1996). For all the GLMs used, graphical representations revealed a good fit between the randomized quantile residuals and the normal probability plot. Furthering the analysis of the residuals, we made for each response at each date a spatial representation of the simplest model residuals over the set up. This representation permitted us to locate any spatial structuration of the residuals that would have revealed a non-independence of the number of insects caught in traps located close together.

Finally, to avoid all possible bias caused by interference inside a taxon (like aggregation behavior), all GLM analyses were carried out again in the presence/absence of analyzed taxa in each trap by using a binomial link function. The results (not shown) are similar to those obtained when using insect counts, thus supporting the conclusions presented herein.

Results

Identification and Quantification of Volatiles The gas chromatograpy–mass spectrometry (GC-MS) analysis of volatiles trapped with SPME revealed a relatively simple profile essentially composed of sulfides (Fig. 2). The main volatile compound identified was dimethyl disulfide (DMDS). Other compounds such as dimethyl trisulfide, dimethyl tetrasulfide, 2-4-5-trithiahexane, and 2-4-dithiapentane were found in smaller amounts, sometimes as traces, or not at all, depending on the sample.

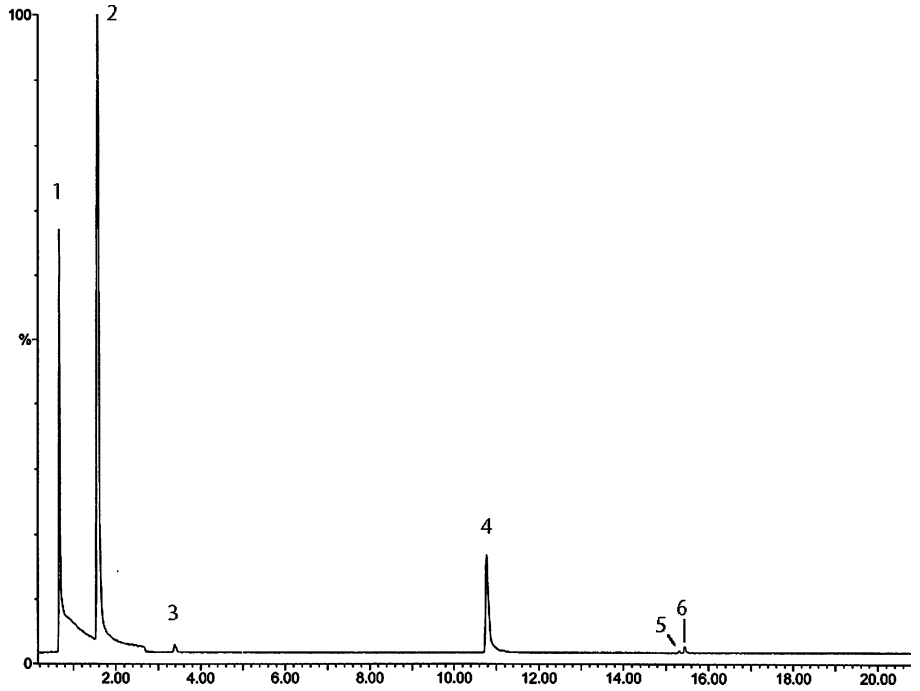


Fig. 2 Typical chromatogram of odors emitted by the rotten part of *Brassica napus* roots heavily infested by *Delia radicum* larvae. 1: SPME injection artifact; 2: DMDS; 3: 2-4-dihiapentane; 4: 2-4-5-trithiahexane; 5: unknown compound; 6: dimethyl tetrasulfide. Refer to the text for details

Among these volatiles, we chose to work with DMDS for several reasons: (1) In all cases, DMDS appeared to be one of the major compounds; (2) As an end product of tissue degradation and because of the high content of sulfur-containing compounds in this genus (notably all glucosinolates), DMDS is likely to be emitted by all attacked Brassicacae roots, regardless of the species infested. Thus, it could be a reliable marker of the presence of *D. radicum*.; (3) DMDS is known as an end product of Alliaceae tissue degradation (Auger et al. 1989b) and could be emitted by these plants also when attacked by *D. antiqua*, a host and prey of both staphilinids studied in this paper.

The quantitative measure of DMDS in infested roots revealed a highly variable concentration. The quantity of DMDS emitted per gram of fresh product in 1 hr, measured on six samples ranged from 0.5 to 38.2 nl, with a mean of 11.2 nl g^{-1} hr^{-1}.

Quantity of DMDS Emitted by the Traps The quantity of DMDS emitted by the traps we used decreased with time. With an initial volume of 2 µl pure DMDS inside the trap, the quantity emitted during the first 6 hr after opening was in the same range as what was emitted by 1 g of infested roots: 38.5±13.2 nl/hr (±SE) between the first hour and the second hour, down to 8.0±0.86 nl/hr (±SE) between the fifth hour and the sixth hour. After 24 hr, only traces were recovered.

Insects Caught in the Traps The 270 traps caught a total of 473 arthropods distributed as follows: 123 *A. bipustulata*, 83 *A. bilineata*, 68 other staphylinids, 155 carabid beetles, 26 other coleopterans (essentially Halticinae), 11 dipterans (all belonging to the genus *Delia*),

one ant, six Arachnids, plus hundreds of springtails (Hexapoda: Collembola) that were not taken into account. Of these arthropods, 32.8% were caught in the traps that contained infested roots, whereas only 6.6% were caught in control traps. The remaining 60.6% of individuals trapped were divided among the traps that contained one of the four DMDS concentrations. For each treatment, a certain number of traps did not catch any of the species we were interested in (N=33, 26, 15, 11, 24, and 12 for T, D002, D02, D2, D20, and P, respectively).

A. bilineata A total of 83 *A. bilineata* adults were caught (0.31±0.06 per trap). The treatment effect was highly significant (Table 1, Fig. 3). Traps containing 0.02 or 0.2 μl of DMDS did not catch more *A. bilineata* than control traps. Traps containing 2 or 20 μl of DMDS caught a higher number of *A. bilineata* than control traps and were not significantly different from each other. Traps that contained infested root caught more *A. bilineata* than all the other treatments (Fig. 3).

The row and column effects were both significant, but without any obvious general pattern (none of the internal or the external rows or columns was responsible for this effect). This observation was consistent with the spatial representation of residuals that did not reveal any structuration.

Although odorous traps captured more females (N=48) than males (N=33), the sex ratio was not biased (*binomial test*, P=0.120), and did not differ from that obtained from control pitfalls for the same week (58 females and 44 males, *Chi-square* test between attractive traps and control pitfalls: χ^2=0.030; 1 *df*, P=0.861).

A. bipustulata A total of 123 *A. bipustulata* adults were caught (0.46±0.05 per trap). The treatment effect was highly significant (Table 1, Fig. 4). Traps that contained only 0.02 μl of DMDS did not catch more insects than control traps, whereas traps that contained 0.2, 2, and 20 μl of DMDS or traps that contained infested roots did. Traps with 2 μl of DMDS caught a higher number of insects than other DMDS concentrations and were not significantly different from traps that contained infested roots.

The influence of the date was significant for *A. bipustulata* (43 individuals caught between June 28 and 29, 52 between June 29 and 30, and 38 between July 4 and 5). As for *A. bilineata*, there was no effect of the interaction between the date and the treatment.

Table 1 General linear model (treatment + date + row + column + treatment × date) results summarizing the effect of the different factors on the number of *Aleochara bilineata*, *A. bipustulata*, and carabid beetles caught per trap

	A. bilineata			*A. bipustulata*		Carabid beetles	
	df	Deviance	P(>|Chi|)	Deviance	P(>|Chi|)	Deviance	P(>|Chi|)
Treatment	5	80.47	< 0.001	44.16	< 0.001	40.77	< 0.001
Date	2	5.72	0.06	7.46	0.024	20.04	< 0.001
Row	24	64.54	< 0.001	32.05	0.126	28.08	0.26
Column	27	57.80	< 0.001	31.50	0.251	55.88	< 0.001
Treatment × Date	10	9.56	0.48	17.36	0.067	14.38	0.16

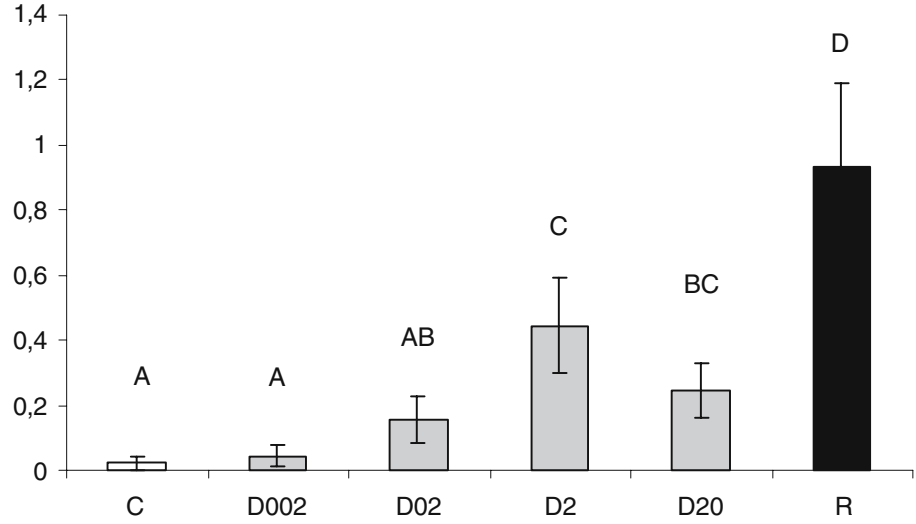


Fig. 3 Mean number of *Aleochara bilineata* caught per trap for each treatment ± SE. (C: control; D002: 0.02 µl DMDS; D02: 0.2 µl DMDS; D2: 2 µl DMDS; D20: 20 µl DMDS; R: rotten part of infested root). Different letters indicate a significant difference between treatments. α=0.05

The row and column position of the traps had no effect (Table 1), in agreement with the spatial representation of residuals.

Sex ratios in the odorous traps were biased toward females (74 females and 49 males, *binomial test*, P=0.030), but this was not caused by odors as a similar bias was observed in control pitfalls for the same week (control pitfall: 81 females and 56 males, *Chi-square* test between attractive traps and control pitfalls, χ^2=0.002; 1 *df*, P=0.965).

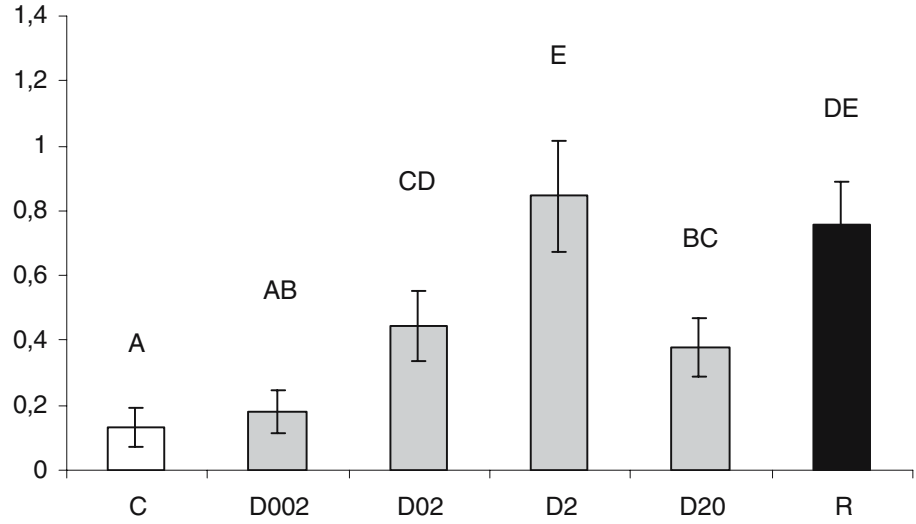


Fig. 4 Mean number of *Aleochara bipustulata* caught per trap for each treatment ± SE. (C: control; D002: 0.02 µl DMDS; D02: 0.2 µl DMDS; D2: 2 µl DMDS; D20: 20 µl DMDS; R: rotten part of infested root). Different letters indicate a significant difference between treatments. α=0.05

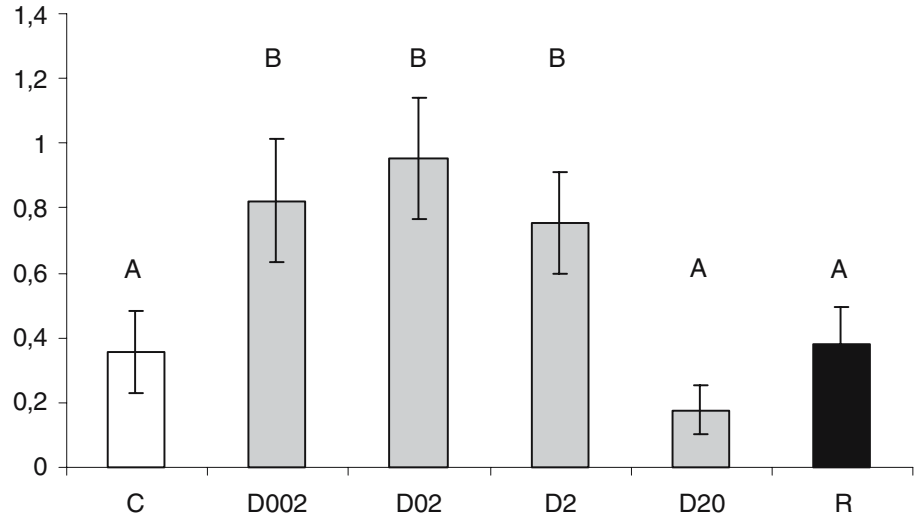


Fig. 5 Mean number of carabid beetles caught per trap for each treatment ± SE. (C: control; D002: 0.02 μl DMDS; D02: 0.2 μl DMDS; D2: 2 μl DMDS; D20: 20 μl DMDS; R: rotten part of infested root). Different letters indicate a significant difference between treatments. α=0.05

Carabid beetles A total of 155 carabid adults was caught (0.57±0.06 per trap). All were identified *a posteriori* as *Bembidion tetracolum* (*N*=91) or *Bembidion lampros* (*N*=62) with the exception of one *B. quadrimaculatum* and one *Microlestes minutulus*. The treatment effect was highly significant (Table 1, Fig. 5). Surprisingly, the traps with the highest concentration of DMDS (20 μl) or naturally infested root odor had no significant effect on carabids, whereas traps with lower concentrations (2, 0.2 or even 0.02 μl of DMDS) caught more carabid beetles than control traps.

The influence of the date was highly significant, particularly because of the twice lower number of insects caught on July 4–5 (*N*=27, compared to *N*=67 and *N*=61 captured on June 28–29 and 29–30, respectively). Nevertheless, there was no effect of the interaction between the date and the treatment.

The column effect was significant, although the row effect was not. As in the case of *A. bilineata*, however, no obvious pattern appeared. Furthermore, the observation of the spatial representation of residuals revealed no structuration.

Discussion

Field bioassays revealed the ecological importance of DMDS in the orientation behavior of the main predators of *D. radicum* eggs and larvae. This compound, frequent in sulfur-containing plants, was emitted in large amounts by brassica roots heavily infested by *D. radicum*. Our results show that DMDS attracts the staphylinids *A. bilineata*, *A. bipustulata,* and the carabids *Bembidion tetracolum* and *B. lampros*. Concentration of the pure molecule seems to play a role, influencing the response of the different predators. To our knowledge, this is the first field study that demonstrates that the concentration of one monomolecular odor influences the orientation behavior of different arthropod predators that belong to the same guild. These results shed new light on the use of general cues by foraging insects, and

could also be helpful for enhancing biological control of *D. radicum*, which feeds on open field crops.

To date, the role of DMDS as a carnivorous insect attractant has been shown only in two tritrophic complexes. The first involves cabbage plants (*Brassica oleracea* spp. *capitata*), the phytophagous lepidoptera *Plutella xylostella*, its specialist parasitoid wasp *Cotesia plutellae*, and a generalist predator, the lacewing *Chrysoperla carnea* (Neuroptera) (Reddy et al. 2002). The second involves leek (*Allium porum*), the phytophagous specialist microlepidoptera *Acrolepiopsis assectella*, and its parasitoid wasp *Diadromus pulchellus* (Dugravot and Thibout 2006). In the first study, Reddy et al. (2002) found DMDS in the frass of *P. xylostella* that was attractive to *C. plutellae* and *C. carnea* in a Y-tube olfactometer setup. In the second, the parasitoid wasp *D. pulchellus* was attracted by a blend of odors coming from attacked leeks (that emit thiosulfinates) and odors of *A. assectella* larval frass (Dugravot et al. 2005; Dugravot and Thibout 2006), the latter material being known to emit a high concentration of DMDS (Auger et al. 1989a). Although DMDS appears to be present in interactions that involve sulfur-containing plants and their associate herbivorous species, little is known about its importance as a cue at the third trophic level (i.e., predators and parasitoids). This study is the first carried out in field conditions that demonstrates such a role for this molecule.

As an end product of sulfur-containing tissues, DMDS is likely emitted by all Brassicaceae infested by *D. radicum*, regardless of plant species. Interestingly, it is also an end product of Alliaceae (Auger et al. 1989b), which are frequently grown in the same geographical zones as cole crops and are attacked by *D. antiqua*, another important host (and prey) of both staphylinids studied here. Accordingly, the positive response of *A. bilineata* and *A. bipustulata* toward DMDS could allow them to locate two of their major preys and hosts (i.e., *D. radicum* and *D. antiqua*) regardless of the infested plant involved. Assessing attractiveness of DMDS in *Allium* crops would confirm this hypothesis.

The main difference we found between the two staphylinid species was their reaction toward the full blend of volatiles emitted by *Brassica napus*-infested roots. Whereas *A. bilineata* showed a preference for this more natural and complete odor, *A. bipustulata* was as much attracted by 2 μl of DMDS as by the natural odor source. One hypothesis is that the more specific the resource, the more specific the cues used to locate this resource should be (Vet and Dicke 1992; reformulated by Steidle and Van Loon 2003). Because *A. bipustulata* is more generalist than *A. bilineata*, notably in that its larvae can parasitize pupae of coprophagous and necrophagous flies, it could benefit from using a general cue like DMDS. Indeed, DMDS is not only emitted by decaying plant tissues that contain sulfur, but also by *any* decaying tissue that contains sulfur. Therefore, DMDS is most likely present in the microhabitat of the coprophagous and necrophagous hosts of *A. bipustulata*, thus making it a good general indicator of prey for this *Aleochara* species. It is notable that the "carrion" smell emitted by the inflorescence of some Araceae, which rely on necrophagous insects for pollination, consists mainly of dimethyloligosulfides (i.e., DMDS or dimethyl trisulfide) (Kite and Hetterschieid 1997). The presence of DMDS in decaying carrion could also explain why pure DMDS attracted carabid beetles (which, as generalists, are also necrophagous), while DMDS-containing odors of naturally infested roots did not.

The difference of attractivity of DMDS found between staphylinids and carabids could highlight the importance of the concentration of a cue in the environment for its use by foraging insects. Indeed, widespread volatile cues apparently lacking specificity could have informative properties that guide specialists to their specific resource based on the concentration of the volatile. In our experiments, the responsiveness of the different taxa toward the nonspecific cue DMDS went from a response to a wide range of concentrations

for the generalist carabid beetles to a response to a narrower range of concentrations (close from what is emitted by the natural odor source) for the more specialist *A. bilineata*. Whereas carabid beetles responded to three of the four concentrations tested, *A. bilineata* responded to only two. The dose–response curve of the two staphylinid beetles shows the strongest response centered around what we found to be the natural quantity of DMDS emitted by an infested root, whereas the dose–response curve for carabid beetles seems wider and centered on the lowest concentration of the molecule. These findings are in accordance with the higher specialization of *A. bilineata* and *A. bipustulata* toward *D. radicum*, in comparison with the carabid beetles.

Associative learning (Vet and Dicke 1992; Dicke et al. 1990a; Takabayashi et al. 2006) or the presence/absence of a particular background odor (Bell 1990; Mumm and Hilker 2005; De Boer and Dicke 2006) could also play a role in attraction toward a nonspecific chemical cue like DMDS. Indeed, predators in a cabbage field could learn by experience that DMDS is often associated with sites where prey can be found. Also, DMDS could only be used when it is part of a full blend of decaying root odor. The extent to which learning or background odors may influence the response of the predators studied herein to different concentrations of DMDS remains to be investigated.

Because of the decreasing rate of volatile emission in our traps, and because of possible differences in the diel activity rhythms of the different predators, it remains possible that the different species caught in the same treatment were faced with different DMDS concentrations when trapped. To avoid this potential bias, future experiments should use a method that ensures the regular diffusion of odors, or work on a shorter time period. Another potential bias that could have influenced the distribution of the different species among the treatments is interference between these species. It is notable that carabid and staphylinid beetles were found in different traps most of the time. However, even if such avoidance occurred, the number of traps was high enough to allow the species to avoid each other without limiting their choice for their preferred treatment. In fact, for each treatment an important number of traps did not catch any insect.

In spite of the potential bias, the setup gave precise and reproducible results. GLM statistics permitted a clear discrimination between the different treatments, even with a low number of insects caught per trap. Furthermore, spatial analysis of residuals allowed checking the validity of assumptions, like the local character of attraction and the independence of data between two adjacent traps. Indeed, neither the effects of the row and columns where the traps were placed nor the spatial representation of residuals revealed any differentiation between the margins and the center of the setup (which could have been the case if individuals had been attracted from outside the setup). Likewise, there was no spatial structure of the residuals (i.e., spatial aggregation of smallest or highest residuals near a particular treatment) as would have been found if traps had influenced each other. Because of its high selectivity, the number of simultaneous comparisons allowed, and the power of the statistical analysis that can be conducted, this setup seems effective in testing local attraction for small ground-dwelling beetles (smaller than 3 mm) in realistic field conditions.

The use of our understanding of the chemical ecology of tritrophic interactions to enhance biological control practices is a motivating approach. To date, only the studies of James (2003, 2005) have shown the possibility of using synthetic VOCs for this purpose. By adding general cues such as methyl salicylate or *cis*-3-hexen-1-ol in plots, the number of flying beneficial arthropods caught in sticky traps increased. The results presented in this paper are the first that show attractivity of beneficial ground-dwelling beetles toward a synthetic odor in the field. Attractiveness of DMDS toward the main natural enemies of *D. radicum* could be useful for biological control. However, before DMDS can be used in the

field, we need to define precisely the conditions under which it is attractive to predators and how it will influence their efficiency to control *D. radicum* populations. Ongoing lab and field experiments should give us such information.

Acknowledgments The authors thank the staff of the experimental station of La Rimbaudais, Saint-Méloir-des-Ondes, France, for providing experimental fields; Romain Rouchet for help on the field experiment; Yannick Outreman and Manuel Plantegenest, Agrocampus, Rennes, France, for advice on statistical analysis; Roxina Soler, NIOO-KNAW, Heteren, The Netherlands, for useful comments on a previous version of the manuscript. This work was supported by a PhD grant to A. Ferry from the Region Bretagne and benefited from the financial help of the GDR d'écologie chimique CNRS no. G2827 for the chemical analysis.

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Introduction

Mutualisms are widespread, ranging from plant pollinators to microbes hosted by plant roots or animal guts. Their long-term persistence is striking given that selection should favor individuals that increase their fitness at the expense of the mutualist partner. To counter this evolutionary instability, each partner must be able to prevent overexploitation by the other. Identifying the mechanisms that prevent overexploitation of one species by its partner is fundamental to understanding the evolutionary stability of mutualisms.

Some plants are highly specialized in pollination and use insects whose larvae feed on the developing seeds of the host plant. An increase in insect fitness (i.e., more eggs laid and more seeds eaten) is costly for plant seed production, creating a conflict of interest between the interacting species. The strategies evolved by plants to prevent overexploitation by seed predators include morphological traits, such as long styles preventing oviposition in figs, or yucca fruit morphology preventing seed access to yucca moth larvae, and fruit abortion in yucca and senita cactus (Pellmyr and Huth 1994; Holland and DeAngelis 2006). Surprisingly, although the chemical response of plants to damage from phytophagous insects through constitutive (Wittstock and Gershenzon 2002) and induced (Bennett and Wallsgrove 1994; Roda and Baldwin 2003) secondary compounds is an acknowledged phenomenon, the role of plant chemical defenses in regulating plant-seed eating pollinator mutualisms has so far been underexplored.

The European globeflower *Trollius europaeus* is exclusively pollinated by six species of flies (genus *Chiastocheta*), whose larvae feed only on globeflower seeds. The female deposits one to several eggs, on or between the carpels at various flower stages, depending on the species (Pellmyr 1989; Després and Jaeger 1999). The larva from each species has a specific location in the globeflower complex fruit, which is composed of several follicles (hereafter referred to as carpels). After hatching, the larvae develop on seeds throughout fruit maturation. Previous experiments have shown that the number of seeds eaten by each larva sharply decreases when the number of larvae per fruit increases, despite the fact that seeds are still available (Jaeger et al. 2001; Després and Cherif 2004). Moreover, larval mass is negatively correlated with the number of larvae co-developing in an infrutescence. Such density-dependent larval growth inhibition might result from the induction of a chemical defense by the attacked plant.

Among the secondary metabolites, phenolic compounds have traditionally been considered to play an important role in plant–insect interactions (Harborne 1999; Simmonds 2003) and can act as attractors, feeding deterrents, or toxic compounds (Summers and Felton 1994; Takemura et al. 2002). They have been identified as active inhibitors of larval growth for the larvae of corn earworm (*Heliothis zea*) (Elliger et al. 1980), autumnal moth (*Epirrita autumnata*) (Ossipov et al. 2001), and fall armyworm (*Spodoptera frugiperd*a) (Johnson et al. 2002; Urrea-Bulla et al. 2004). To determine whether plant phenolics could be involved in the control of seed predation in the *Trollius europaeus–Chiastocheta* system, we compared the phenolic profiles of unparasitized fruits vs. fruits naturally parasitized by an increasing number of larvae in two alpine sites at different altitudes. We found that a flavonoid is overproduced in the carpel walls of parasitized fruits. We quantified the variation of this compound in several *Trollius* species, in various plant organs, and at different developmental stages. Mechanical damages were performed on unparasitized plants to test for the specificity of the plant response. We discuss the potential implication of this tissue-specific, density-dependent induced chemical compound in the evolutionary stability of this obligate plant-seed eating mutualism.

Material and Methods

Study Species The European globeflower *Trollius europaeus L.* (Ranunculaceae) is a hermaphroditic arctic-alpine perennial species growing in moist meadows (Després and Jaeger 1999; Jaeger et al. 2000). Two study sites were chosen in two mountain massifs in the French Alps (Chartreuse and Galibier), 100 km apart. These two sites (Cherlieu, 950 m a.s.l. 5°46′E 45°18′N and Galibier 2,300 m a.s.l. 6°24′E 45°04′N) below and above the tree line, respectively, were previously extensively studied (Jaeger and Després 1998; Hemborg and Després 1999; Jaeger et al. 2000, 2001; Després et al. 2007).

Phenolic Profiles of Parasitized vs. Unparasitized Fruits To obtain unparasitized fruits, flower buds were bagged with a nylon mash bag and hand pollinated with pollen from several donors. Parasitized and unparasitized fruits were sampled 3 wk after flowering in 2003, June 18th in Cherlieu (N=15 and N=30, respectively) and July 8th in Galibier (N=11 and N=36, respectively). Back in the laboratory, the fruits were dissected immediately, and the developing larvae were counted and discarded. From each fruit, three parts (the carpel walls, seeds, and floral receptacle) were carefully separated and individually stored at −18°C until they were analyzed. A first subset of 22 fruits from Cherlieu (eight without larva, seven with one larva, and seven with several larvae) was submitted for an analysis of total phenolic compounds, which was carried out on each of the three fruit components. Based on preliminary results (see below), the entire set of carpel walls was submitted for complementary analysis (phenolic acids and flavonoids). This individual procedure and the tiny size of some of the samples meant that dry weight could not be measured. Therefore, all results were expressed in fresh weight (FW). Five of the larvae were pooled, stored at − 18°C, and analyzed for phenolic content.

Each sample was weighed and separately extracted twice with 50 ml of an ethanol–water (50:50) mixture under reflux. After evaporation under vacuum, the residue was redissolved in a precise volume of distilled water, filtered (0.45 μm), and used for Folin-Ciocalteu colorimetric determination of total amounts of phenolic compounds (Marigo 1973).

Aliquots (20 μl) of the ethanolic solution were used for high performance liquid chromatography (HPLC) analysis on a RP C18 μBondapak column, 4.6×250 mm, and gradients were monitored by a Waters 600 Controller. Solvent A was acetic acid 0.5% in distilled water and solvent B acetic acid 0.5% in acetonitrile. Phenolic acids were determined by using a linear gradient from 0 to 20% of B in A in 45 min, with a 1.5 ml min^{-1} flow. The spectra were recorded with a Waters 996 PDA in the 200- to 400-nm range. Chromatographic peak areas were determined at 280 nm. Areas were compared after multiplying the area by the volume of extraction corresponding to each carpel. Standards of common phenolic acids and aldehydes were obtained from Sigma. Flavonoids (including adonivernith) were separated with an isocratic flow (1.5 ml min^{-1}) of 20% of B in A. The peak areas were recorded at 354 nm. Standards (luteolin, orientin, homorientin) were obtained from Extrasynthese (Lyon, France). The standards were prepared in methanol as stock solutions at 1 mg ml^{-1}.

Structural Elucidation of Flavonoid Liquid chromatography-mass spectrum (LC-MS) analysis: ten ethanolic extractions were pooled, concentrated, and used for mass determination with electrospray ionization MS (ESI-MS) on the positive mode [M+H]. Mass spectra were recorded from a ZQ Waters Mass detector (electrospray voltage 4.2 kV) coupled to a μBondapak RP C18.

Nuclear magnetic resonance (NMR) analysis: To obtain sufficient amounts of flavonoid, 75 g FW of frozen flowers were extracted with methanol (70:30) under reflux for 30 min. The methanolic extract was filtered and evaporated. The aqueous residue was successively extracted with petroleum ether and ethyl acetate. The ethyl acetate fraction was evaporated

to dryness, redissolved in MeOH, and fractionated by gel filtration using Sephadex LH-20 (Amersham, 3.5×50 cm, MeOH, 2 ml min^{-1}) to obtain 40 fractions. The fractions were monitored by HPLC, and fractions containing adonivernith were combined and dried.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-400 and a Varian Unity+ 500, respectively, in MeOH-d_4 as solvent at 303 K. Standard pulse sequences and parameters were used for the experiments (COSY, DEPT, HMQC, HMBC, TOCSY, and NOESY). Chemical shift references were given from the solvent resonances at 3.30 ppm for ^{1}H and 49 ppm for ^{13}C.

Adonivernith Occurrence in T. europaeus and in Other Trollius Species To assess the distribution of adonivernith within the whole *T. europaeus* plant, six flowering plants (without larvae) were collected in Cherlieu in 2004 and dissected into seeds, floral receptacle, leaves, staminodias, sepals, carpels, and stamens. The six samples were pooled and extracted by ethanolic mixture as described above.

The kinetics of the synthesis of adonivernith by the whole flower was followed over five developmental stages (N=3), from green bud to old fruits (21 d after flowering). The entire organ (flower or fruit) was analyzed for flavonoid content as previously described. Fruits of three other species of *Trollius of Asian Origins Trollius chinensis*, *T. ledebouri*, and *T. pumilus* were collected during summer 2005 in the Alpine Garden of the Joseph Fourier station (Col du Lautaret, 2,100 m a.s.l.) and stored frozen (−18°C) until analysis.

Mechanical Damage Mechanical damage was inflicted in two ways: first, low-intensity wounds were inflicted in Cherlieu by piercing the carpel walls or the stem (to measure a systemic response) with a needle; and second, high-intensity wounds were inflicted in Galibier by incising the carpel wall with a scalpel (five incisions). In Cherlieu, 59 young floral buds were bagged and hand-pollinated 1 wk later. Ten holes were pierced on the stems of 19 flowers and on the carpel walls of 21 other flowers; this treatment was repeated 1 wk later. Three-week-old fruits were collected, and stored at −18°C until they were analyzed. In Galibier, incisions were made on 25 flowers. The wounded flowers and 21 control flowers (at the same developmental stage) were bagged to prevent infestation. Fruits were collected 11 d later and kept frozen (−18°C) until they were analyzed.

Statistical Analysis Data were checked for normality of distribution and homogeneity of variances (Kolmogorov–Smirnov and Levene tests). Where the data did not satisfy these criteria, nonparametric tests were performed (e.g., Spearman's rank correlations R_s, Mann–Whitney tests). Otherwise, t tests were used to compare parameter of parasitized and intact fruits. To examine whether globeflowers from the two sites differed in their chemical response to increasing larval numbers, the slopes of the correlations between the number of larvae and the flavonoid amounts were compared in Cherlieu and Galibier by performing an analysis of covariance (ANCOVA), testing for the effect of population, larvae number, and interaction on the amount of flavonoid. A significant interaction indicates that the slopes are different within each population.

Results

Phenolic Composition of Parasitized versus Unparasitized Fruits Concentrations of total phenolic compounds did not differ significantly in seeds from unparasitized vs parasitized fruits collected in Cherlieu (8.6±0.7 vs. 9.1±1.0 mg g^{-1} FW) (Mann–Whitney test, U=54,

$P>0.05$), or in the floral receptacle (7.3±1.0 vs. 8.5±0.6 mg g^{-1} FW) ($U=39$, $P>0.05$). In contrast, the carpel walls of fruits infested by one or several larvae exhibited higher concentrations of total phenolic compounds (7.8±0.4 mg g^{-1} FW) than intact, uninfested fruits (5.7±0.5 mg g^{-1} FW) ($U=28$, $P<0.01$). As a single larva contained only 0.024 mg of total phenolic compounds, this increase is not caused by larvae excretion (the larvae were discarded during dissection).

Further analyses were, therefore, carried out on the carpel walls only. Because the number of larvae developing in a globeflower increased with carpel number and with the mass of the carpels (Fig. 1) ($R_s=0.477$, $P<0.01$, $N=29$ at Cherlieu; $R_s=0.603$, $P<0.01$, $N=26$ at Galibier), we preferentially assessed phenolic synthesis by measuring the absolute amount per fruit rather than the concentration.

The two populations did not differ in terms of total amounts of phenolics in unparasitized carpels (Mann–Whitney test, $U=63$, $P>0.05$), but the concentration was higher in Galibier (7.8±0.6 mg g^{-1} FW) than in Cherlieu (5.4±0.3 mg g^{-1} FW) ($U=30$, $P<0.01$). This was because of the higher carpel mass in Cherlieu ($m=0.31\pm0.04$ g) compared to Galibier ($m=0.18\pm0.03$ g). The total amount of phenolics per fruit (Fig. 2) was correlated to larval number per fruit in each of the two study populations ($R_s=0.562$, $P<0.05$, $N=45$ at Cherlieu; $R_s=0.705$, $P<0.01$, $N=47$ at Galibier). The highest amount per fruit (6.0 mg) was recorded in Galibier in a fruit parasitized by six larvae and was four times higher than the mean amount observed in unparasitized fruits from the same site (1.3±0.2 mg). To determine which fraction was responsible for the increase of total phenolics with the increasing number of larvae, we carried out a chromatographic analysis adapted to detection of phenolic acids and flavonoids.

Seven compounds were recorded when using the chromatographic conditions for phenolic acid determination (Table 1). These compounds could not be identified as any of the most common phenolic aglycones used as standards, probably because they occur as glycosides. Because unparasitized and parasitized fruits did not differ in the relative area of each compound (Mann–Witney tests, $P>0.05$), no further attempt was made to identify them.

Five flavonoids had areas high enough to be recorded on the chromatogram (Table 2). Concentrations of the five compounds significantly increased with larval number per fruit in Galibier, whereas the concentration of only one flavonoid (F3) significantly increased in Cherlieu. This compound, characterized by a retention time of 5.4 min, also exhibited the

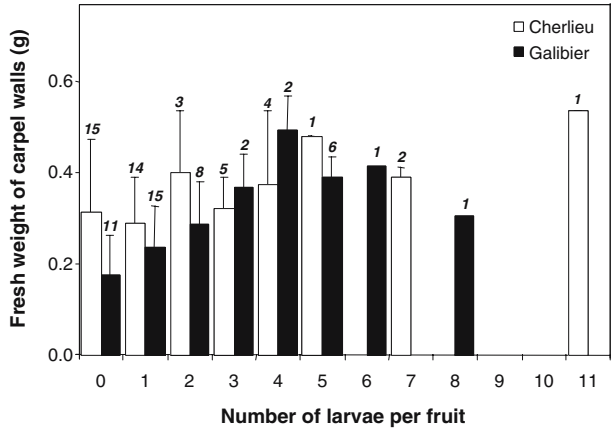


Fig. 1 Fresh weight (mean + SE) of carpel walls according to the number of larvae per fruit for the two populations: Cherlieu ($N=45$) and Galibier ($N=47$). Sample numbers (italics) are indicated above the bars

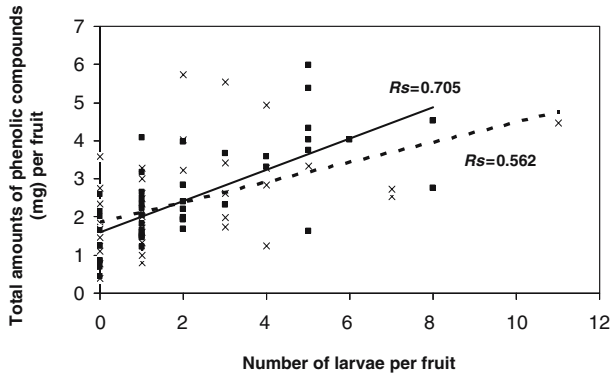


Fig. 2 Spearman correlation (*Rs*) between total amounts of phenolic compounds and the number of larvae in fruits of *T. europaeus* from low altitude (Cherlieu, x, dashed line) (N=45) and high altitude (Galibier, ■, solid line) (N=47) sites. Total amounts of phenolic compounds (Folin-Ciocalteu) were expressed in milligrams of gallic acid equivalent per fruit

highest area (68–79% of the total area). It was selected for structural identification and further identified as adonivernith (see below). Moreover, total amounts of phenolics and amounts of adonivernith were highly correlated (R_s=0.786 and 0.691, P<0.001, in Cherlieu and Galibier, respectively). F2 and F4 have the same UV spectra as F3, indicating the same flavone basis.

In unparasitized fruits, the amount of adonivernith per fruit was slightly higher in the population at low altitude (Mann–Whitney test, U=45, P=0.051) because of larger fruits, but no differences were detected when concentrations were compared between the two populations (U=68, P>0.05). In contrast, in parasitized fruits, the amounts of adonivernith (and the concentrations) were higher at the high altitude site: 117±13 μg in Galibier vs. 62± 6 μg in Cherlieu (U=245, P<0.005). The highest amount of adonivernith (331 μg) was observed in Galibier in a fruit parasitized by five larvae, and represented 17 times the mean amount (19 μg) in unparasitized fruits. In Cherlieu, the highest increase was only by a factor of 4 (from 38 μg for the mean amount in unparasitized fruits to 151 μg for the highest amount). The stronger chemical response to larval infection of plants at the high-altitude site was confirmed by significantly different slopes for the correlation between the number of larvae and the amounts of adonivernith induced in Galibier and in Cherlieu: R_s 0.851 vs. R_s

Table 1 Peak areas (mean ± SE) of phenolic acid like compounds obtained by chromatographic analysis (HPLC-DAD) of intact (N=5) and parasitized (N=5) carpel wall extracts

Phenolic Compounds	Peak Areas[a]	
	Without Larvae	With N>1 Larvae
P 1[b]	537±137[c]	1325±159
P 2	858±194	2021±177
P 3	574±87	825±102
P 4	782±260	1262±128
P 5	1071±289	1419±300
P 6	1867±403	914±273
P 7	1057±276	1241±244

[a] Peak areas were recorded at 280 nm.

[b] The order of the phenolic compounds in this table followed their order of elution.

[c] No significant differences were detected between the two groups.

Table 2 Retention time (rt), proportion of the peak areas of flavonoids (percent of total area), and spearman correlation coefficients (*Rs*) between number of larvae and peak areas for the five flavonoids obtained by chromatographic analysis (HPLC-DAD) of parasitized carpel wall extracts from cherlieu (*N*=45) and Galibier (*N*=47) populations

Flavonoids	R_t (min)	Percent Total Area [a]		R_s [b]	
		Cherlieu	Galibier	Cherlieu	Galibier
F1	3.5	8	4	0.010	0.491**
F2	4.9	6	5	0.346	0.694***
F3	5.4	68	79	0.479*	0.851***
F4	7.2	16	9	0.117	0.543***
F5	8.5	2	3	-0.156	0.532**

[a] Peak areas were recorded at 350 nm.

[b] Levels of significance: *P<0.01; **P<0.005; ***P<0.001

0.479, respectively (Fig. 3) (ANCOVA, larval number: $F_{1,\ 85}$=39.01 P<0.001; population $F_{1,\ 85}$=4.32, P=0.04; larval number by population interaction: $F_{1,\ 85}$=11.77, P<0.001).

Structural Elucidation of Adonivernith The UV spectra recorded from HPLC-DAD analysis gave λ_{max} 256(sh), 270, 350 nm, characteristic of the flavone luteolin. The positive ion mode MS spectra gave an $[M+H]^+$ ion at *m/z* 581, indicating a possible derivative of luteolin containing one hexose and one pentose substitute. ^{13}C and ^{1}H NMR were required to allow characterization of the linkage and configuration of the sugar moieties. The compound was identified as luteolin 8-β-D-glucopyranosyl-2″-*O*-D-xylopyranoside (or orientin 2″-*O*-D-xylopyranoside), and the spectral data are in good agreement with respective literature data (Wagner et al. 1975; Markham et al. 1987). This compound was previously named adonivernith from its first identification in *Adonis vernalis* (Hörhammer et al. 1960).

Mechanical Induction of Phenolic Metabolism To measure the specificity of the phenolic response as previously demonstrated, we carried out two experiments with mechanical damage of different intensities on flowering globeflowers and analyzed the production of adonivernith in the carpel walls (Fig. 4). Compared to the control plants, no differences were observed between the amount of adonivernith in the carpel walls pierced by a needle (*t* test, t=0.766, P>0.05), nor in those more seriously injured by using a scalpel (t=1.714, P>0.05), nor when the floral stem was pierced (t=0.563, P>0.05). None of these wounds influenced carpel mass.

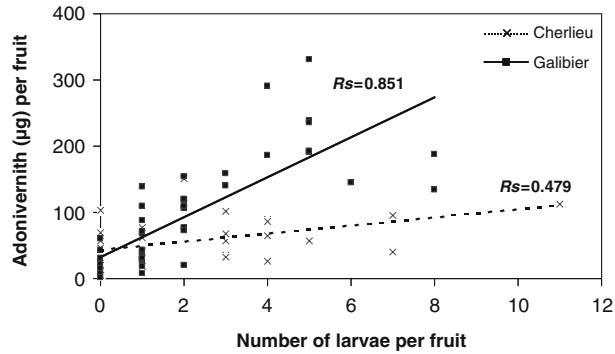


Fig. 3 Spearman correlation (*Rs*) between adonivernith amounts and the number of larvae in fruits of *T. europaeus* from low altitude (Cherlieu, x, dashed line) (*N*=44) and high altitude (Galibier, ■, solid line) (*N*=47) sites. Adonivernith amounts were expressed in micrograms of luteolin equivalent per fruit

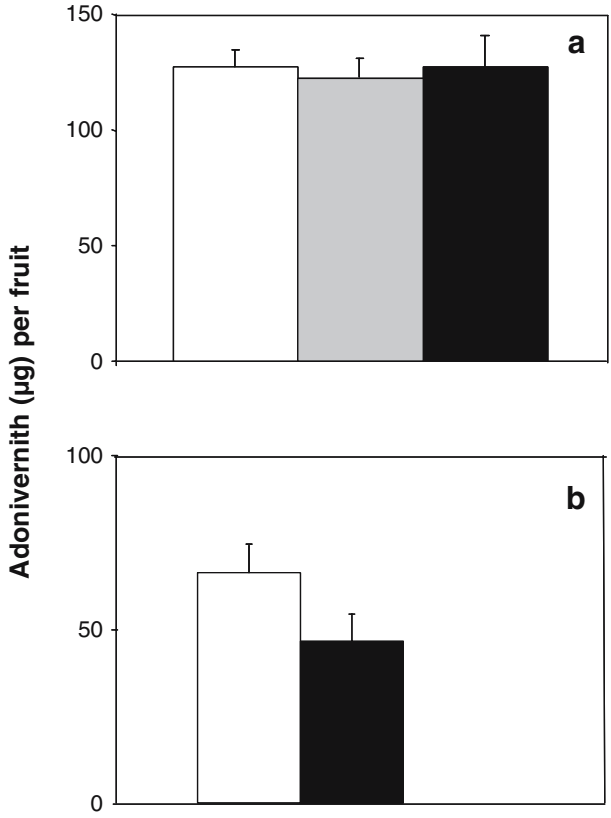


Fig. 4 Amounts (mean + SE) of adonivernith in carpel walls of *T. europaeus* mechanically damaged. (**a**) In Cherlieu, wounds (10 holes) were inflicted with a needle (= low wound) on the carpel (■ black, *N*=19) or on the stem (▒ gray, *N*=21) and compared to control plants (□ white, *N*=19). (**b**) In Galibier, wounds (five incisions) were inflicted with a scalpel (= severe wound) on the carpel (■ black, *N*=21) and compared to control plants (□ white, *N*=25). Adonivernith amounts were expressed in micrograms of luteolin equivalent. No statistically significant differences were observed

Adonivernith Occurrence in T. europaeus and Other Species The fruits of three other *Trollius* species (*T. chinensis*, *T. ledebouri*, and *T. pumilus*) did not contain adonivernith in detectable amounts. However, *T. chinensis* extracts contained two compounds characterized by close retention time (4.6 and 6.1 min, respectively) and similar UV spectra when compared to adonivernith, indicating the likely occurrence of the luteolin flavone.

In *T. europaeus*, adonivernith was not present in the seeds and the floral receptacle, but was found in all the other floral parts and in the leaves (Table 3). Its concentration in the yellow sepals (3.9 mg g^{-1}) was especially high. Except for staminodias, adonivernith was generally more concentrated in all the flowering parts than in unparasitized mature carpels previously analyzed (0.11 mg g^{-1}). The decrease in adonivernith concentrations during the maturation process (Table 4) was also observed when the complete flowers at different developmental stages (from buds to mature fruits) were analyzed.

Discussion

This study is the first to show that larvae of a specific mutualist can induce a chemical plant response, suggesting a means of partner control to prevent overexploitation. This is also one of a few studies on induced plant defenses that have been conducted in field conditions,

Table 3 Concentration of adonivernith in the different organs of *T. europaeus* plants (N=6, pooled) collected at 1-day flower stage

Plant Organs	Adonivernith (mg of luteolin eq./g)
Leaf	0.83
Floral receptacle	_[a]
Sepal	3.88
Staminodia	0.09
Carpel wall	0.24
Stamen	0.20
Seed	_[a]

[a] Below the detection limit

rather than in controlled environment. Therefore, it was not possible to set up a control for the abiotic environmental conditions (e.g., UV intensity) nor for the biotic stresses other than *Chiastocheta* parasitism (e.g., pathogens) that might influence plant phenolic metabolism (Koricheva et al. 1998; Reifenrath and Muller 2007). For instance, adonivernith concentrations in intact carpel walls ranged from 52 to 394 μg g^{-1} in Cherlieu, and from 7 to 355 μg g^{-1} in Galibier. Large variability in plant secondary metabolite production, presumably under both genetic and environmental determinism, is a well-documented phenomenon, and is consistent with previous findings that the chemical profiles of the roots of *Caltha leptosepala* and *Trollius laxus* are highly influenced by habitat characteristics (Kuhajek et al. 2004). Despite these field constraints, individual fruit analyses showed that *Chiastocheta* larval development enhances phenolic metabolism in the carpel walls of the globeflower, and that this increase in content of phenolics is mostly caused by a C-glycosyl flavone that we identified as the rare adonivernith.

The occurrence of luteolin and of C-glycosylflavones in *T. europaeus* leaves has previously been reported (Gonnet 1981; Lebreton 1986), but this is the first report of its presence in several organs of this plant. In the *Trollius* genus and in the closely related genus *Adonis* (Després et al. 2002), the classical flavonols and *O*-glycosyl flavonols are largely replaced by the less widespread flavones and C-glycosyl flavones (Jensen 1995). Adonivernith was first isolated from *Adonis vernalis* (Hörhammer et al. 1960) and more recently from *Trollius macropetalus* (Liu et al. 1992), but was also reported in the Poaceae *Setaria italica* (Harborne and Baxter 1999). *T. chinensis* and *T. ledebouri* have been more extensively studied than *T. europaeus*, and C-glycosyl flavones structurally related to adonivernith (like vitexin and orientin) have been found in their flowers (Liu et al. 2004;

Table 4 Adonivernith concentration (mean ± SE) (N=3) of *T. europaeus* flowers at different developmental stages

Developmental Stage of the Flower	Adonivernith (mg of luteolin eq./g)
Green floral bud	2.38 ± 0.76^{a}
1-day flower	1.09 ± 0.49^{ab}
7-day flower (nonpollinated)	0.71 ± 0.24^{b}
7-day flower	0.64 ± 0.11^{b}
14-day fruit	0.08 ± 0.02^{c}
21-day fruit	0.04 ± 0.04^{c}

[a] Different superscript letters indicate significant differences (Mann–Whiney test, P<0.05) between the developmental stages

Zou et al. 2005; Li et al. 2006). However, we found no adonivernith in these two species, nor in *T. pumilus*, indicating species specificity.

Adonivernith has been found in significant amounts in most organs of *T. europaeus* plants, especially in the sepals where it probably contributes to the bright yellow coloration. Phenolic compounds found in flowers play both attractive and defensive functions (Gronquist et al. 2001) leading to the question of the significance of the correlation between mutualism-linked traits (attractive colors) and antagonism-linked traits (defensive compounds) (Herrera et al. 2002). However, adonivernith induction by larval infestation seems restricted to the carpel walls. Other minor flavonoids also participate in the globeflower-induced response, but their contribution to the total flavonoid production is low. At least two of them are structurally close to adonivernith, i.e., luteolin-based C-glycosyl flavones, indicating possible metabolism intermediates.

Another argument to support the specificity of this chemical plant response to larval infestation is the lack of induced response to mechanical damage, whatever the intensity and the location of the wounding. *Chiastocheta* larval regurgitant might be required to trigger plant-induced response as found in other plants (Alborn et al. 1997). The increase in adonivernith production observed in parasitized fruits might be an efficient defense mechanism if it affects the survival or growth of the larvae. Another luteolin-based C-glycosyl flavone, maysin, induced after infestation of corn by caterpillars of corn earworms (*Helicoverpa zea*) reduces larval growth (Elliger et al. 1980; Wiseman et al. 1993). Such a growth inhibitor effect by adonivernith on *Chiastocheta* larvae has not been demonstrated. So far, experimental bioassays have been limited by the difficulty of breeding *Chiastocheta* larvae in the laboratory. Preliminary experiments have nevertheless been conducted during short time periods: *Chiastocheta* larvae exposed on paper imbibed with carpel extracts for 4 hr did not exhibit increased mortality, compared to larvae on watered paper. In the same manner, choice experiments showed no deterrence of those extracts. The observations in the field that larval survival is independent from the number of larvae developing in a fruit (Jaeger et al. 2001), and that individual larval fresh mass at the end of development decreases according to that number, although seeds are not limited (Després and Cherif 2004), supports the hypothesis that the induced compound inhibits larval growth rather than kills the larvae.

The present study also shows that the increase in adonivernith production is proportional to the number of larvae developing within the infrutescence. Recent empirical and theoretical studies suggest that density-dependent processes control mutualistic interactions (Holland and DeAngelis 2006). For example, yucca moth density was shown to be regulated by selective abortion of highly parasitized fruits (Pellmyr and Huth 1994). These results were later challenged by several studies that showed that fruit abortion was random in yucca (Addicott 1998; Shapiro and Addicott 2004); however, although random, fruit abortion might limit and regulate yucca moth population growth through density-dependent recruitment. Similar density-dependent population level processes are believed to occur in the senita cactus–senita moth mutualism (Holland and DeAngelis 2006). The density-dependent chemical response of globeflowers might limit fly population growth at densities where the interaction is still mutualistic, which could contribute to the interaction stability.

We also found that adonivernith overproduction is tissue specific: only the carpel walls of parasitized fruits show an increase in the chemical defense, whereas the seeds and the floral receptacle remain unchanged. Such carpel-specific accumulation of phenolic compounds has been observed in *Hypericum calycinum*, raising the question of the development of specific chemical defensive profiles by plant carpels (Gronquist et al. 2001). Although larvae of all *Chiastocheta* species feed on *T. europaeus* seeds, the larvae

from each species are spatially partitioned within a single infrutescence, either in the floral receptacle (the first ovipositing species), or mining their way through the inner or outer carpels (the five remaining species) (Després and Jaeger 1999; Pompanon et al. 2006). The first species infesting the fruit occupies the only undefended part of the fruit (adonivernith-free refuge), whereas all the other species appear to be more exposed to adonivernith. The tissue-specific differential induction of adonivernith after larval infestation creates a heterogeneous chemical environment within a single fruit ("chemical niches") that might have selected for different fly resistance strategies, such as escape to a phenolic-free refuge for the first visitor, and evolution of metabolic resistance for later visitors. This hypothesis remains to be tested by evaluating the differential resistance of early vs. late *Chiastocheta* species to adonivernith exposure. If verified, this mechanism might explain the unusually large number of congeneric flies coexisting on *T. europaeus* fruits.

Acknowledgments This work was partly supported by the CNRS (GDR 2877). We thank Charlotte Tollenaere for assistance in the field and the UMS 2925 Joseph Fourier Alpine Station for logistics support provided and for giving us access to the exotic *Trollius* species. We thank C. Bosso from CERMAV for LC/mass analysis and Kim Barrett for correcting our English.

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may be impaired by direct anthropogenic disturbances. For example, Wolf and Moore (2002) found that exposure to the pesticide metolachlor rendered crayfish unable to detect food sources and also elicited inappropriate behavior to other chemicals that could jeopardize their survival. Human impact can also alter the concentration of naturally occurring substances, which may additionally impair chemical recognition. This study examines the potential effect of the naturally occurring substance humic acid (HA). HA is a by-product of degrading organic matter and is increased by processes such as eutrophication (Thomas 1997).

Dissolved organic matter and humic substances constitute the majority of dead organic matter (Steinberg and Münster 1985; Thomas and Eaton 1996; Thomas 1997; Wetzel 2001). HA is a ubiquitous, naturally occurring organic component in aquatic environments associated with agricultural manure and other organic wastes (Atiyeh et al. 2002; Kappler and Haderlein 2003). Elevated levels of HA in aquatic habitats are of particular interest because of their apparent effect in impairing the recognition of conspecific sexual pheromones (Hubbard et al. 2002; Fisher et al. 2006). Mesquita et al. (2003) found that humic acids adsorb relatively water-insoluble organic substances such as steroidal pheromones, reducing their biological availability to organisms that normally detect these chemicals as part of their communication. Hubbard et al. (2002) proposed that the steroids adsorb onto the surface of humic acid microvesicles that form in water, rendering the pheromone effectively unavailable for olfactory detection by *Carassius auratus* and other teleosts in natural environments.

Female swordtail fish (*Xiphophorus birchmanni*) lose their preference for conspecific males over heterospecific males in water with elevated levels of HA (Fisher et al. 2006). This impairment of chemical communication may have implications for the evolutionary fate of many species because of the potential for interspecific hybridization.

The zebrafish, *Danio rerio* (family Cyprinidae), is an important vertebrate model system in many areas of biological research. Zebrafish are native to freshwater streams and rice paddies in the Ganges River of eastern India, Bangladesh, and Burma, where they are likely exposed to many naturally occurring organic substances (Mcclure et al. 2006; Spence et al. 2006). Environmental effects reported from many of the rice-producing countries include soil degradation, water contamination with nitrogen, eutrophication, and increased ammonia and other nitrogen trace gas emissions (Li 1995; Robertson et al. 2000). Additionally, from 1980 to 2000, use of synthetic nitrogen fertilizers in East Asian rice-producing nations has increased from approximately 4 to 10 Tg [Food and Agricultural Organization (FAO) 2002] as part of a transition from more traditional to industrialized, broad-scale rice production. As zebrafish use chemical communication in the context of reproductive suppression and stimulation (Van den Hurk et al. 1987; Delaney et al. 2002; Gerlach 2006) and kin recognition (Mann et al. 2003; Gerlach and Lysiak 2006), the chemical environment is likely to affect transmission and reception of chemical stimuli. In this study, we investigated the ability of adult *D. rerio* to discriminate between conspecific and heterospecific chemical cues in the presence and absence of HA, as well as their habitat preferences for water with and without HA.

Methods and Materials

Animal Care and Housing We tested wild-type adult zebrafish that were raised together in the zebrafish facility at the Marine Biological Laboratory, Woods Hole, MA, USA. Fish were housed in an Aquatic Habitats tank system with a 14 L:10 D cycle and were fed twice daily with dry fish pellets and live *Artemia salina* nauplii. We used adult zebrafish from the

F_1 generation of wild-type individuals that originated from several pet shops and breeding centers in the USA, with the objective of obtaining out-crossed strains with higher genetic variability than fish from the same source.

Choice Tests Trials were performed in a choice flume (Fig. 1) powered by a peristaltic pump. The flume allows a laminar flow separation of two water stimuli (Atema et al. 2002; Mann et al. 2003; Gerlach and Lysiak 2006). The water height was maintained at approximately 1.5 cm, and uniform unidirectional flow was maintained at a constant rate of 106 ml/min or 6.2 cm/s). Dye tests were conducted to confirm proper separation of the two different water flows.

During odor choice tests, a single test fish was placed into the flume to acclimatize for 5 min before measurements began. The test consisted of two 5-min periods where odor stimuli were presented on alternate sides of the flume to control for possible side bias of the fish. After the first 5-min, another 1-min acclimation time was allowed when stimuli were alternated in the flume, and the test continued for another 5 min. From position records taken every 10 s, we calculated how often each fish selected a given stimulus. Water association time is widely used as a measure of preference in studies of kin recognition, shoaling preferences, and mate choice in laboratory studies on zebrafish (Rosenthal and Ryan 2005; Gerlach and Lysiak 2006; Gerlach et al. 2007) and poeciliids, including swordtails (see Wong and Rosenthal 2005 and references therein). We calculated the difference between the total number of observations in which a test fish was in the lane of the flume associated stimulus A or B, and tested whether that difference was significant from zero by using the Wilcoxon matched-pairs signed-ranks (WSR) test. If the fish did not have a preference for one of the odor stimuli or was unable to detect a difference between them, we would expect a random distribution on either side of the flume, which would result in no (zero) difference in distribution frequency between treatments. A nonparametric Van der Waerden chi-squared test was used to analyze differences between experiments (discussed below). Analyses were performed with SAS 3.1 software (SAS Institute).

We performed three sets of tests. First, we tested the response of male and female zebrafish to untreated water (dechlorinated tap water, pH 7.0) vs water containing 200 mg/l supplemental HA. Second, we presented female zebrafish with water containing conspecific cues vs water containing heterospecific cues (goldfish, *Carassius auratus*). Heterospecific water was created by isolating one goldfish with a body mass of approximately 35 g to a standing tank

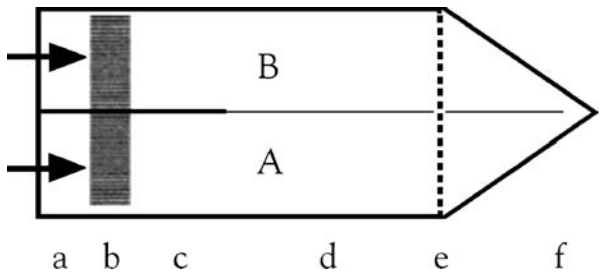


Fig. 1 Diagram of the choice flume. **a** Water inflow area, **b** collimator to homogenize turbulent flow, **c** barrier-separated channels, **d** area of flume where water columns remain separated (*fine dotted center line*), **e** screen to contain test subjects, and **f** outflow channel. *A* The left side of the flume and *B* the right side of the flume, when oriented toward the start of the flow. The dimensions of the flume are 33.6×7.1×6.5 cm (after Mann et al. 2003)

of untreated water. Conspecific water was made by isolating a single male zebrafish with a body mass of approximately 1 g to a separate standing tank. Tanks were filled with the same volume of untreated water (7 l) with airstone aeration 24 h before being used in the flume experiments. The heterospecific water was then diluted 35 times in untreated water to correct for body mass difference between the goldfish and the zebrafish. Third, we repeated the second set of experiments, this time treating both conspecific and heterospecific water stimuli with HA (Fluka cat. #53680) for a final concentration of 200 mg/l HA (Fisher et al. 2006). Several workers, including Malcolm and Mccarthy (1986) have pointed out differences between commercial humic acids, like the ones used in the present study, and HA derived from natural sources. Specifically, commercial HA have considerably higher carbon-to-nitrogen ratios and high ash contents (Malcolm and Mccarthy 1986). These factors are unlikely to fundamentally affect the behavior of HA with respect to chelating chemical signals or acting as antagonists to chemoreceptors.

To prepare HA-treated water, we measured 200 mg of HA and placed the amount in a 1,000-ml volumetric flask. The flask was filled with 500 ml of untreated water, magnetically stirred for about 15 min, and the final volume was brought up to 1,000 ml. The mixture was transferred to a bottle that was capped and shaken vigorously for 5 min, then set aside for 1–2 h before being used. The solution was shaken thoroughly once more before being used in flume trials.

Results

In odor choice tests that assayed habitat preference, zebrafish associated more with untreated water over HA-treated water (WSR test, $z=59$, $N=19$, $P=0.015$; Fig. 2).

Female zebrafish tested in water without supplemental HA preferentially associated with conspecific cues over heterospecific cues (WSR test, $z=162.5$, $N=25$, $P<0.001$; Fig. 3).

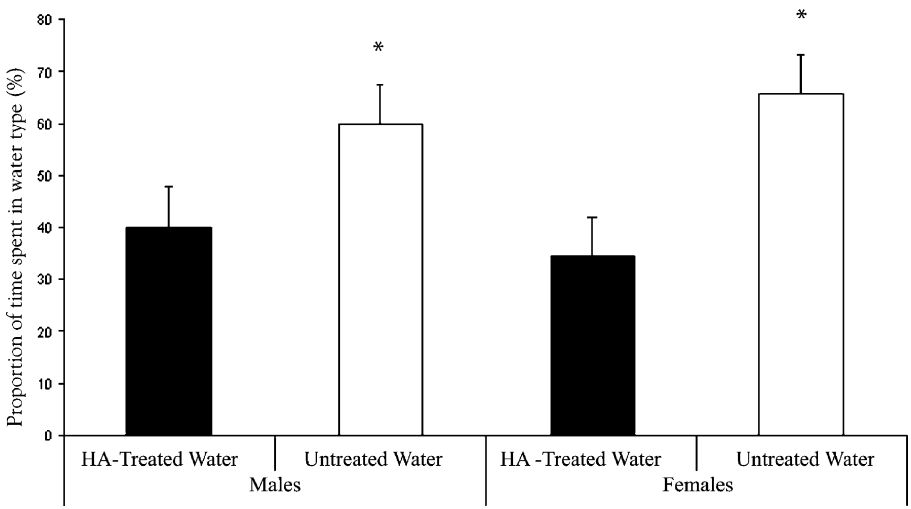


Fig. 2 Response (mean±SE) of zebrafish to untreated water vs water containing supplemental HA. *Error bars* indicate the standard error of means, and an *asterisk above a column* indicates a significant difference between untreated water and HA-treated water (*$P<0.02$)

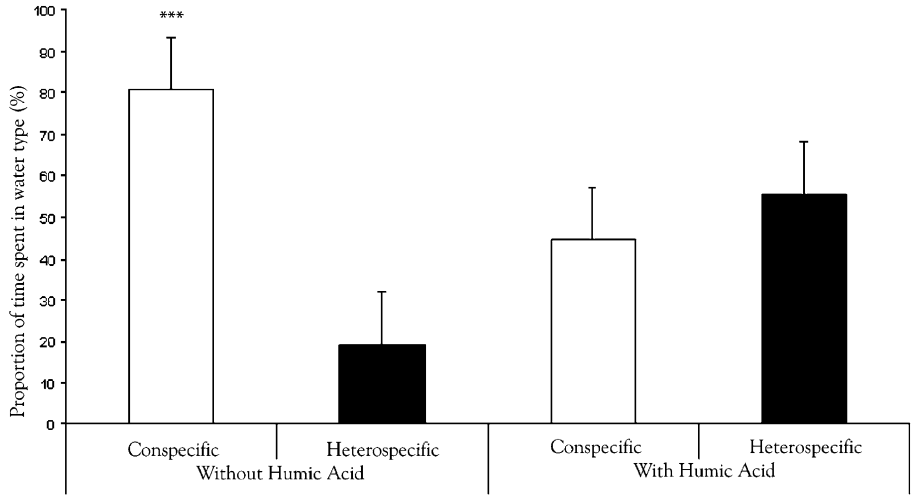


Fig. 3 Preference of female zebrafish (mean±SE) for conspecific (*black*) vs heterospecific (*white*) stimuli in water with and without 200 mg/l HA. *Error bars* indicate standard error of means, and an *asterisk above a column* indicates a significant difference between association with conspecific and heterospecific cues in untreated water (***$P<0.001$)

When 200 mg/l HA were added to stimuli containing conspecific and heterospecific odor cues, zebrafish failed to show a preference ($z=63$, $N=25$, $P=0.09$; Fig. 3). The observed association for conspecific cues was significantly reduced in water containing supplemental HA. Zebrafish spent 82±3% of the observation time in proximity to conspecific odor stimulus in water without supplemental HA, but when tested in HA-treated water, they associated 44±3% of the trial with the conspecific odor cue (van der Waerden test, $\chi^2=26.3$, $df=1$, $P<0.001$; Fig. 3).

Discussion

Our results suggest that humic acid can interfere with recognition of conspecific chemical cues in zebrafish. While zebrafish showed a strong association with olfactory cues of conspecifics vs heterospecifics, they did not differentiate between these odor cues when tested in conditions with elevated levels of HA. These findings suggest that humic acid either reduces zebrafish preference for conspecific cues or reduces their avoidance from heterospecific cues. Our experimental design, however, does not allow us to discriminate between these two hypotheses; nevertheless, the biological significance is the same in either case: Humic acid alters zebrafish behavior toward chemical cues.

Elevated levels of HA in the environment as a byproduct of anthropogenic disturbance could potentially lead to misidentification of shoalmates and hybridization (Fisher et al. 2006). Zebrafish are sympatric with other danionin fishes over part of their range (Mcclure et al. 2006) and shoal assortatively with respect to species-typical visual cues (Engeszer et al. 2004; Rosenthal and Ryan 2005). The olfactory channel may be critical in social communication, particularly, when transmission of visual information is poor (Rosenthal and Lobel 2006).

Chemical cues may also influence habitat choice. Zebrafish strongly avoided water containing HA in favor of clean water. The chemical environment may be an important

determinant of habitat choice. Given the physiological (Yang et al. 2002; Steinberg et al. 2004) and sensory (Fisher et al. 2006) costs of HA exposure, selection may favor individuals who actively avoid high HA concentrations. These findings have important implications for species living in aquatic habitats with high levels of pollutants and agricultural runoff. In response to such environmental disturbances, many organisms may avoid areas important for survival, such as foraging areas and mating grounds (Sprague 1962; Geckler et al. 1976). High HA concentrations may drive organisms into suboptimal but less polluted habitats.

Several studies have demonstrated the deleterious effect of HA on a variety of organisms. High levels of HA in a natural habitat may have effects on multiple physiological and behavioral functions. HA can decrease olfactory response to pheromones in goldfish (*Carassius auratus*) and prevent pheromones from binding properly to chemoreceptors (Hubbard et al. 2002; Mesquita et al. 2003). Most known fish sexual pheromones are either steroids or prostaglandins (Stacey et al. 2003; Yambe et al. 2006), and their hydrophobic properties may make them more susceptible to HA binding. It remains to be seen how olfactory communication is maintained in fish that live in areas with naturally high concentrations of HA (Rosenthal and Lobel 2006).

Acknowledgment We thank Jelle Atema, Carley Schacter, and Michelle Gaona for the discussion and advice.

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of a ripe fruit odor—either in combination with visual cues or when presented alone. Fruit bats also used fruit odors to distinguish between ripe and unripe fruits. By using gas chromatography (GC) and GC/mass spectrometry (MS), a total of 16 main compounds were identified in the ripe fruit odor of *Ficus hispida* and 13 in the ripe fruit odor of *Ficus scortechinii*—including alcohols, ketones, esters, and two terpenes. Additional compounds were also recorded in *F. hispida*, but not identified—four of which also occurred in *F. scortechinii*. Total scent production increased in both species when fruits ripened. Both natural and synthetic fruit odors resulted in feeding attempts by bats, with no feeding attempts elicited by unscented controls. Reaction rates to natural fruit odors were higher than those to synthetic blends.

Keywords Seed dispersal syndromes · Frugivory · Old world tropics · Co-evolution · Olfaction · Foraging ecology · Moraceae · Chiroptera · Pteropodidae · Fruit scent · Sensory ecology

Introduction

Observations in tropical rain forests have revealed a broad dietary overlap between birds and mainly diurnal mammals when feeding on figs (Breitwisch 1983; Leighton and Leighton 1983; Shanahan et al. 2001a). This has led to the widespread belief that interactions between figs and their vertebrate seed dispersers are relatively generalized, as characterized by large crops of low-quality fruits consumed and dispersed by diverse and taxonomically disparate groups of frugivores. However, more recent studies on the feeding ecology of fruit bats, in both Neo- and Paleotropical rain forests, have revealed a distinct subset of fig species that are consumed and dispersed almost exclusively by nocturnal mammals—especially bats (Kalko et al. 1996; Korine et al. 2000; Shanahan and Compton 2001; Shanahan et al. 2001a; Hodgkison et al. 2003).

In contrast to diurnally dispersed figs, which are often characterized by visually conspicuous fruit displays, particularly bright colors that stand out against surrounding vegetation (e.g., orange and red), nocturnally dispersed species are often characterized by distinctive odors (Lambert 1987, 1989; Lambert and Marshall 1991; Kalko et al. 1996; Korine et al. 2000; Shanahan and Compton 2001; Shanahan et al. 2001a; Hodgkison et al. 2003). These adaptations in fruit display and fruit chemistry correspond closely with the sensory capabilities and foraging behavior of both groups of disperser—as birds and diurnal mammals have well-developed color vision, and locate fruits primarily by sight (Dominy et al. 2003; Smith et al. 2006; Schaefer et al. 2007), whereas fruit bats and other nocturnal mammals have an acute sense of smell, and locate fruits primarily by olfaction (Rieger and Jakob 1988; Laska 1990a, b; Acharya et al. 1998; Thies et al. 1998; von Helversen et al. 2000; Luft et al. 2003; Sanchez et al. 2004, 2006; Korine and Kalko 2005). Thus, many fig species are likely to be more specialized in their interactions with frugivores than previously thought (Kalko et al. 1996; Shanahan et al. 2001b; Hodgkison et al. 2003). The chemical compounds that mediate the interactions between figs and their vertebrate seed dispersers are currently unknown, although high levels of ethanol, which are often associated with overripe fruits, may act as a feeding deterrent to fruit bats (Sanchez et al. 2004).

The goals of this study were to investigate the fruit odors of two bat-dispersed fig species (*Ficus)* in the Paleotropics, in relation to the foraging behavior of fruit bats (Pteropodidae). We tested the following hypotheses: 1) fruit odor plays a critical role for the detection and selection of ripe figs by fruit bats; 2) bat-dispersed figs are characterized by

the same, or similar, chemical compounds; and 3) total scent production, in bat-dispersed figs, increases significantly when fruits ripen. We performed bioassays to test the effect of both natural and synthetic fruit odors on the foraging behavior of the short-nosed fruit bat, *Cynopterus brachyotis* (Müller), an important disperser of fig species within the study area (Hodgkison et al. 2003).

Methods and Materials

Study Site The field work was conducted at Kuala Lompat (3°43′N, 102°17′E), within the Krau Wildlife Reserve, Pahang, Peninsular Malaysia between March 2005 and August 2006. The Krau Wildlife Reserve consists of a large area of old-growth forest, which rises from 50 m (asl) at Kuala Lompat, to over 2000 m at the summit of Gunung Benom. The vegetation at Kuala Lompat can be described as lowland evergreen mixed dipterocarp forest (Raemaekers et al. 1980). However, compared to many other lowland dipterocarp forest sites in Malaysia, most of which have now been cleared for agriculture, this site is unusually rich in large Leguminosae. The site is also extremely rich in strangler and free-standing figs (*Ficus* spp.), with 41 species reported to date, at least 25 of which are consumed by birds and 10 by bats, with little overlap (Lambert 1987, 1989; Lambert and Marshall 1991; Hodgkison et al. 2003). The majority of fig species consumed and dispersed by birds at Kuala Lompat are characterized by visually conspicuous fruit displays that are produced as a result of distinct color changes upon ripening, often following the transition from green or yellow-green to bright orange or scarlet red (Lambert 1987, 1989). Figs consumed and dispersed by bats, by contrast, tend to be visually less conspicuous to humans, typically yellow-green, or dark red-brown, but are often characterized by the production of faint or strong odors when ripe. Several bat-dispersed fig species at Kuala Lompat are also caulicarpic, producing fruits on woody knobs or long runners that grow directly from the main trunk (Hodgkison et al. 2003).

The bat-dispersed species selected for sampling in the present study contrast in both color and odor production when ripe. *Ficus hispida* Linn. F. has a distinct color change upon ripening, from green to yellow, and emits a strong fruity odor as an attractant. *Ficus scortechinii* King, by contrast, exhibits only a slight color change upon ripening, from dark red-brown to slightly paler red-brown, and produces an odor, which, to a human nose, is almost completely imperceptible.

Fruit Odor Sampling Fruit odors of bat-dispersed figs were sampled in the field by using dynamic headspace adsorption techniques (Dobson et al. 2005; Kalko and Ayasse in press). Both ripe and unripe fruits were removed from plants and placed inside glass sample chambers, all of which were connected by equal lengths of silicon tubing to a single battery-operated pump (SKC, type 224). Air drawn into each glass sample chamber through a single inlet was filtered and cleaned of atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (Orbo-32, Supelco), and plugged with silanized glass wool (Raguso and Pellmyr 1998). Air drawn out of each chamber, through a single outlet, was sampled for volatile fruit odor compounds, by a sorbent tube containing a thin layer of activated charcoal (CLSA-Filter 1.5 mg). Both the sorbent tubes and charcoal filters were connected to the glass sample chambers by short lengths of Teflon tubing.

In total, four glass sample chambers were used to collect fruit odors during each sampling session. This allowed up to three fruits to be sampled simultaneously, along with a blank control. Each sampling session was conducted at night (between 1930 and 0500 hours)

with a flow rate of 100 ml min^{-1} per chamber and a total sampling time of 4 hr for *F. hispida* and 5 hr for *F. scortechinii*.

After sampling, all sorbent tubes were wrapped in aluminum foil, labeled, and sealed in airtight glass jars, with Teflon-coated lids, and then stored at −18°C. The samples were eluted the following day with 0.040 ml of 10:1 pentane/acetone. Eluted samples were sealed in small airtight borosilicate glass specimen tubes and returned for storage at −18°C.

After each sampling session, all glassware was thoroughly cleaned three times with ethanol (Absolute Alcohol, Hayman Ltd., Essex, UK), acetone (LiChrosolv, Merck, Darmstadt, Germany), and pentane (SupraSolv, Merck), and placed in a sterilizing oven set at 150°C for 1 hr. Sorbent tubes were cleaned three times with ethanol, dichloromethane (LiChrosolv, Merck), and pentane, and then wrapped in aluminum foil and stored for future use in airtight glass jars with Teflon-coated lids.

Chemical Analysis of Compounds For quantitative analyses, 0.1 μg of *n*-octadecane was added as an internal standard to each of the eluted fruit odor samples collected by dynamic headspace adsorption (see above). All samples were analyzed with an HP5890 Series II gas-chromatograph (Hewlett-Packard, Palo Alto, CA, USA), equipped with a DB5 capillary column (30 m×0.25 mm i.d.) that used hydrogen as the carrier gas (2 ml min^{-1} constant flow). One microliter of each sample was injected splitless at 40°C. After 1 min, the split valve was opened and the temperature increased by 4°C min^{-1} until reaching a temperature of 300°C.

GC/MS analyses were carried out on an HP 6890 Series GC connected to an HP 5973 mass selective detector (Hewlett-Packard) fitted with a BPX5 fused-silica column (25 m, 0.22 mm i.d., 0.25 μm film thick, SGE). Mass spectra (70 eV) were recorded in full scan mode. Retention indices were calculated from a homolog series of *n*-alkanes. Esters and alcohols were purchased (Sigma-Aldrich, Schnelldorf, Germany) when commercially available. If not, target compounds were synthesized by conventional esterification by using an acid chloride and the respective alcohol under Schotten–Baumann conditions (Autorenkollektiv 1999). α-Bergamotene was identified by comparing the mass spectrum and retention index with the data of a critically evaluated database (Joulain and König 1998).

Behavioral Experiments Six behavioral experiments were conducted. The first set consisted of simple fruit detection tests, designed to assess the relative effect of three cues (color, shape, and odor) on the foraging behavior of captive bats. This involved four separate feeding trials, in which each bat was exposed to the following sequence of treatments: 1) ripe fruits, without modification; 2) unripe fruits, without modification; 3) ripe fruits, wrapped in a triple layer of 15 denier black nylon stocking; and 4) ripe fruit pulp smeared on an adhesive polyester patch and covered with a double layer of black stocking. The second set of experiments were two-choice feeding trials, designed to test the hypothesis that bats use fruit odors to distinguish between ripe and unripe fruits. This involved two separate feeding trials in which each bat was offered the following options: 1) an unripe fruit and a ripe fruit; and 2) two unripe fruits, one of which had been artificially scented with ripe fruit pulp. Bioassays were performed to test the effect of both natural and synthetic fruit odors on the foraging behavior of captive bats.

For bioassays, each bat was presented with a single unripe fig (control) or an unripe fruit that had been artificially scented with either a natural ripe fruit odor sample of *F. hispida* (collected by dynamic headspace adsorption techniques—see above) or a synthetic blend of the available compounds present and identified in the ripe fruit odor of the same species. The composition of the synthetic blend was mixed according to the relative proportions identified by gas chromatography. As the enatiomeric compositions of the chiral compounds were unknown, the synthetic blend used racemic (50:50) mixtures of both

enantiomers. The synthetic blend was diluted and applied to the figs at the same concentration as the dynamic headspace samples, using the solvent pentane. This concentration was equivalent to two fruits sampled for 4 hr. Compounds included in the synthetic blend are listed in Table 1. Control bioassays were also performed with pure pentane with no added ripe fruit odors (Nolte and Mason 1998).

All behavioral experiments and bioassays were conducted in a large flight cage (3×3×2.5 m-width × length × height), with newly captured and, hence, naïve bats. Bats were

Table 1 Comparisons of the relative proportions of 22 compounds present in the ripe and unripe fruit odors of *Ficus scortechinii* and *Ficus hispida*

# Compound	Mean Relative Amount (%)									
	Ficus scortechinii					*Ficus hispida*				
	Unripe (*N*=12)		Ripe (*N*=12)			Unripe (*N*=10)		Ripe (*N*=21)		
	Mean	SD	Mean	SD	*U*[a]	Mean	SD	Mean	SD	*U*[a]
1) Compound A	0.000	0.000	0.000	0.000	72	0.015	0.048	0.124	0.235	61
2) 2-Pentyl acetate[b]	3.948	5.319	0.425	0.611	56	3.137	4.892	13.638	7.884	20***
3) (*E*)-Pent-3-en-2-yl acetate[b]	2.436	8.438	0.000	0.000	66	0.000	0.000	6.966	5.692	5***
4) 2-Heptanone[b]	22.109	20.322	7.238	2.433	57	24.207	24.311	29.135	15.318	70
5) 2-Heptanol[b]	0.398	0.891	10.058	5.557	15***	0.559	1.184	11.937	7.113	7***
6) (*Z*)-Hex-3-enyl acetate[b]	0.000	0.000	0.000	0.000	72	0.298	0.632	0.282	0.469	54*
7) 2-Pentyl butanoate[b]	0.000	0.000	0.006	0.021	66	0.360	0.758	1.256	1.691	37**
8) 2-Heptyl acetate[b]	2.903	4.644	30.203	5.481	0***	2.149	2.383	21.472	14.020	22***
9) 2-Pentyl 2-methylbutyrate[b]	0.000	0.000	0.000	0.000	72	2.093	2.506	0.403	0.708	92
10) Furanoid linalooloxide	2.594	3.229	0.120	0.270	63	3.987	3.053	0.220	0.353	39**
11) 2-Nonanone[b]	0.450	1.492	8.233	1.439	0***	0.372	0.610	2.350	2.701	32**
12) 2-Nonanol[b]	25.923	15.108	12.329	5.171	27**	28.803	18.141	1.163	1.579	0***
13) Unknown Acetate	0.527	0.999	0.003	0.009	58	0.484	0.811	0.462	0.516	56
14) Unknown unsaturated Acetate	0.028	0.096	0.000	0.000	56	4.730	7.588	0.206	0.088	80
15) Unknown Acetate 2	0.700	1.285	0.020	0.038	58	2.127	2.810	0.216	0.361	98
16) 2-Pentyl hexanoate[b]	1.468	1.350	0.125	0.283	37*	1.694	1.179	0.196	0.307	22***
17) (*E*)-Pent-3-en-2-yl hexanoate[b]	0.720	0.974	0.138	0.262	60	1.272	1.072	0.106	0.113	37**
18) 2-Nonyl acetate[b]	5.444	9.740	25.793	9.130	11***	0.108	0.244	1.371	1.771	20***
19) 2-Undecanone[b]	0.354	0.843	0.261	0.247	34*	0.632	1.686	0.619	0.914	43*
20) α-Bergamotene	24.120	24.056	1.393	1.993	1***	11.865	9.260	1.705	4.739	30**
21) Compound B	5.354	6.071	2.371	2.992	52	10.701	7.614	5.189	8.132	51*
22) Compound C	0.524	1.031	1.285	0.947	27**	0.407	0.930	0.986	1.375	31**

[a] Compounds that increase or decrease significantly in their relative proportions when fruits ripen, Mann–Whitney *U* test: **P*<0.05, ***P*<0.01, ****P*<0.001

[b] Compounds included in the synthetic mixture of *F. hispida*

obtained by using ground and canopy mist nets within the study area at Kuala Lompat (Hodgkison et al. 2002; Kunz et al. in press). To facilitate the capture of specific fruit bat species that consume figs, mist nets were positioned adjacent to fruiting fig trees, opened at dusk (c. 1900 hours), and checked every 15 min for captured bats—with one individual bat retained for behavioral experiments or bioassays during each night of sampling. All newly captured bats were introduced to the flight cage on the night of capture, and then left undisturbed, with an abundant supply of food, until the following day. Immediately before dusk the following evening, any uneaten fruits were removed from the flight cage to prevent bats from becoming distracted during experiments.

Behavioral reactions of bats were recorded on videotape with the "nightshot" facility of a Sony digital video camera (Handycam DCR-PC350E). The effect of each treatment was quantified by the presence or absence of four types of behavioral response: circling, approaching, landing, and feeding. An "approach" was differentiated from circling, both by its greater precision in relation to the position of the fruit, and by a perceptible reduction in flight speed. A "feeding" attempt was scored when a bat attempted to bite and remove a fruit. Landing always occurred before feeding, and no fruit bat was observed to remove a fruit while in flight. To motivate the bats throughout the experiments, each bioassay was preceded by a feeding trial with a reward of one ripe fruit (*F. hispida*). Each experiment lasted for 20 min, with each individual bat exposed to up to six behavioral experiments or four bioassays per night. All fruits were presented to the bats in a natural way, by using wires to fix them to the trunks of small trees incorporated into the sides of the cage. To prevent bats from learning the spatial arrangements within the cage, all fruits were presented in different locations during each successive trial (Nolte and Mason 1998). The only artificial light source was an infrared beam emitted from the camera.

When experiments were completed, all bats were recaptured in the flight cage with a hand net, marked with an individually numbered stainless steel ball chain necklace (Ball-Chain Manufacturing Company, NJ, USA), and then released at the site of capture (Kunz and Weise in press). This ensured that no individual bat was exposed to a single experiment or bioassay on more than one occasion.

Data Analysis Total scent production and the relative amounts of each compound produced by the ripe and unripe fruits of *F. hispida* and *F. scortechinii* were compared by using the Mann–Whitney *U* test. The relative amounts of all compounds commonly found in both species of *Ficus* were compared with a discriminant function analysis (DFA). The standardized discriminant function coefficients and the factor loadings of each compound were used to assess the differences between the ripe and unripe fruits of both species. All statistical analyses of fruit odors were performed by using SPSS version 11.0.

The results of the behavioral experiments and bioassays were arranged on 2×2 contingency tables to express the observed frequencies of the four behavioral reactions in response to *n* treatments (i.e., approach vs. no approach × *n* treatments, and feeding vs. no feeding × *n* treatments). These results were analyzed with Fisher's Exact Test of independence (Sokal and Rohlf 1995).

Results

Chemical Composition of Fruit Odors Several odor compounds were identified by GC/MS by comparing their mass spectra and retention times with those of authentic reference

samples. This revealed a series of odd numbered 2-alkanones, 2-alkanols, and their respective acetates, butanoates, and hexanoates (Table 1). Compound 3 (Table 1) showed a mass spectrum similar to that of pent-3-en-2-ol. However, a higher molecular mass, by 42 Da, and a more intense ion *m/z* 43 suggested an acetate. Comparisons with synthetic samples of all possible pentenol acetates revealed this compound to be (*E*)-pent-3-en-2-yl acetate (Fig. 1). The corresponding hexanoate was identified by using the same method. Compound 9 (Table 1) proved to be an ester of 2-pentanol with a C_5-acid. Comparisons with authentic reference samples of 2-pentyl pentanoate, 3-methylbutyrate, and 2-methylbutyrate revealed the natural compound to be the latter. The only terpenes found were the furanoid form of linalooloxide and α-bergamotene. Structures of the identified compounds are shown in Fig. 2.

A total of 16 compounds were identified in the ripe fruit odor of *Ficus hispida*, whereas six additional compounds remained unidentified. Twelve of the identified, and four of the unidentified, compounds also occurred in the ripe fruit of *Ficus scortechinii* (Table 1), producing a reduced but similar bouquet (Fig. 3). 2-Heptyl acetate was particularly prominent in the ripe fruit odors of both species, and accounted for ca. 30% of total scent production in *F. scortechinii*, and 21% in *F. hispida* (Table 1). Other prominent compounds included 2-hepanol and 2-heptanone (Table 1).

Total scent production of ripe fruits was significantly greater than that of unripe fruits (Fig. 4), and included a greater number of compounds (Fig. 3). On average, total scent production per fruit of *F. scortechinii* increased from 0.008±0.017 μg hr^{-1} (mean ± SD) to 0.148±0.162 μg hr^{-1} upon ripening (N=12 in both cases); whereas total scent production per

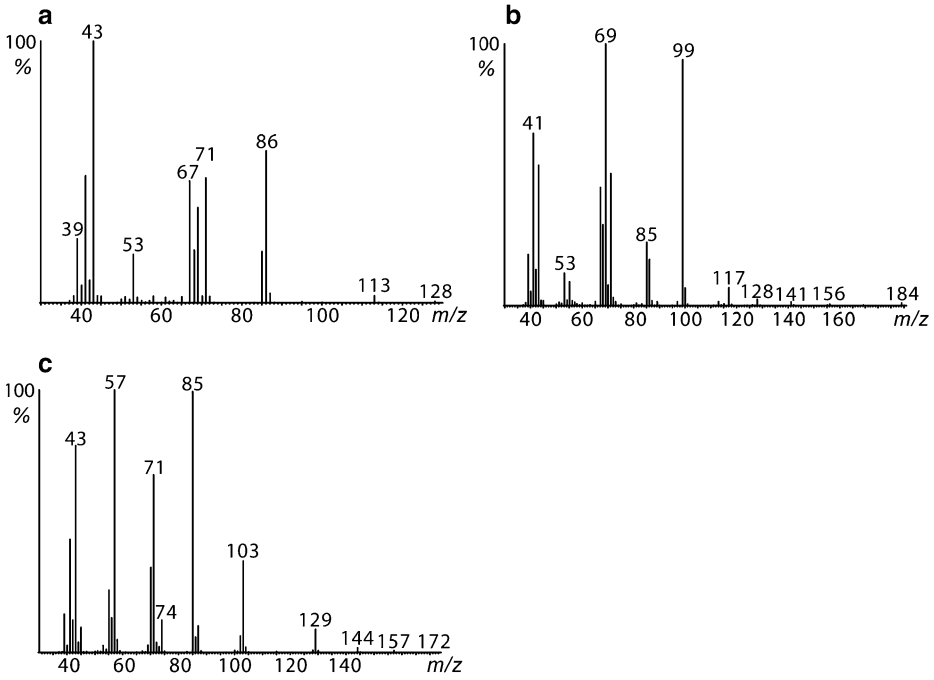


Fig. 1 Mass spectra of (*E*)-pent-2-en-3-yl acetate (**a**), (*E*)-pent-2-en-3-yl hexanoate (**b**), and 2-pentyl 2-methylbutyrate (**c**)

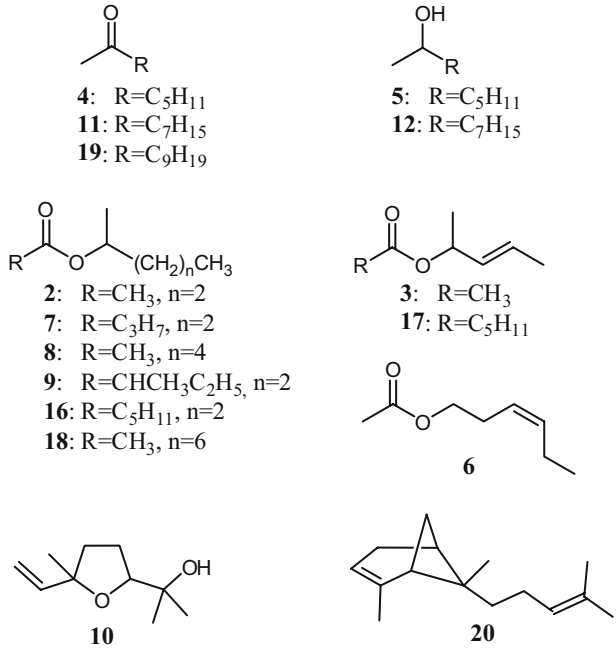


Fig. 2 Structures of compounds identified in the ripe fruit odors of *Ficus scortechinii* and *Ficus hispida*. Numbers refer to entries in Table 1

fruit of *F. hispida* increased from 0.017±0.020 μg hr^{-1} to 0.507±0.546 μg hr^{-1} (N=10 and 20, respectively). Relative amounts of several compounds also increased (e.g., 2-heptanol and 2-heptyl acetate) or decreased significantly (e.g., 2-nonanol and α-bergamotene) when fruits ripened (Table 1). No compounds were associated exclusively with unripe fruits of either fig species.

The discriminant function analysis (DFA) of fruit scents produced four chemically distinct clusters, which corresponded with the ripe and unripe fruits of both fig species (Fig. 5). The first discriminant function, which accounted for 66.5% of the total variance, was mainly important for discriminating between the unripe fruits of *F. scortechinii* and the ripe fruits of *F. hispida* (DF1: Chi-square=267, df=45, P<0.001). The second function, which accounted for 31.6% of the total variance, was mainly important for discriminating between the ripe fruits of *F. scortechinii* and the unripe fruits of *F. hispida* (DF2: Chi-square=129, df=28, P< 0.001). The third function was not statistically significant. Compounds with the highest standardized canonical discriminant function coefficients were pentyl acetate, compound 15, and (*E*)-pent-3-en-2-yl hexanoate (in DF1), and 2-undecanone and compound 22 (in DF2). Of the 54 samples used in the DFA, 53 (98.1%) were correctly assigned to groups—with a cross-validated accuracy rate of 83.3%.

Fruit Bat Foraging Behavior Fruit bats responded to both visual and chemical (olfactory) cues when foraging for fruits. However, in the absence of a ripe fruit scent, the proportion of individuals that approached, or attempted to land or feed on unripe fruits, was significantly lower than on the ripe fruit control (P>0.01, in each case). Of the 20 individual bats tested, only 70% of individuals approached unripe fruits, 15% attempted to land, and only 5% attempted to feed (N=20). By contrast, approach rates of 100% were recorded for all scented treatments, with landing and feeding rates ranging from 95 to 100% (Table 2). In two-choice feeding trials, bats showed a strong preference for ripe fruits over

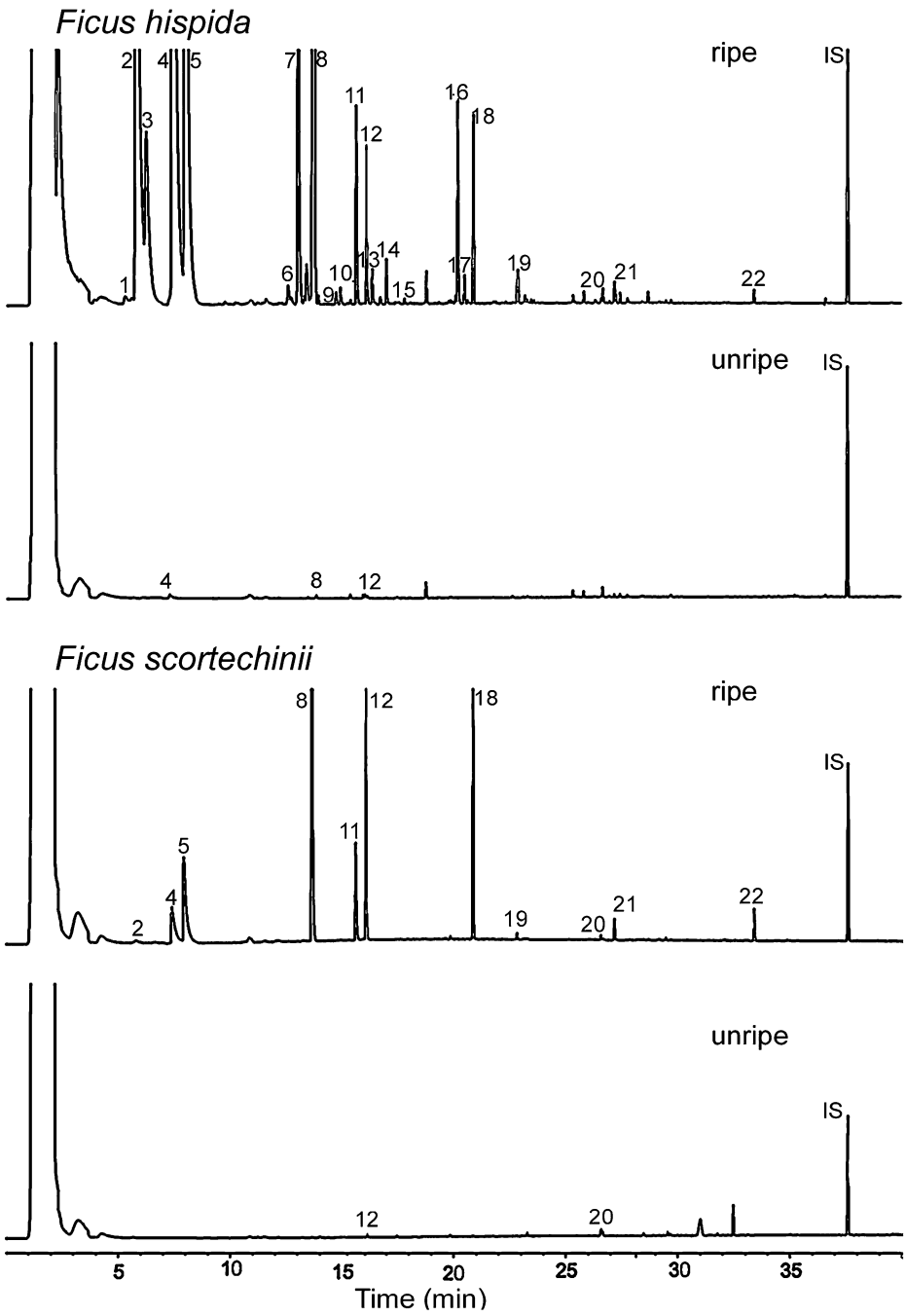


Fig. 3 Gas chromatograms of the ripe and unripe fruit odors of *Ficus hispida* and *Ficus scortechinii*. All labels correspond with the numbers in Table 1. IS = internal standard (octadecane). N.B. compounds 10, 15, 16, and 17 occurred only in trace amounts in *F. scortechinii*

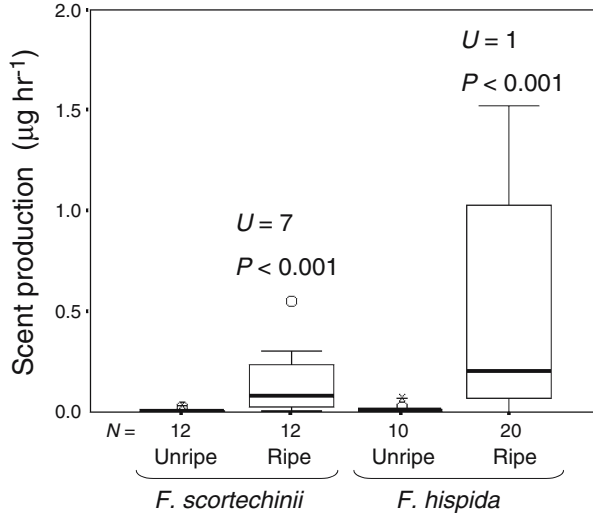


Fig. 4 Total scent production in ripe vs. unripe fruits of *Ficus scortechinii* and *Ficus hispida*. Included are medians, mid-quartiles, 90th and 10th percentiles and outliers. There was a significant increase in total scent production in both species when fruits ripened (Mann–Whitney *U* test)

unripe fruits, and scented (i.e., manipulated) unripe fruits over unscented (i.e., unmanipulated) unripe fruits (Table 3). Responses to ripe and manipulated unripe fruits, smeared with ripe fruit pulp, were not significantly different (P=0.43), with feeding attempts by 100 and 93% of the individuals tested, respectively (Table 3).

Feeding attempts by fruit bats were elicited by both natural fruit odors and synthetic blends. However, a greater proportion of bats reacted to natural headspace samples of *F. hispida* than to synthetic blends of the same species (55 and 15% respectively, N=20, P=0.019). Compared to the two unscented controls, the synthetic blend of *F. hispida* had no significant affect on the proportion of bats attempting to feed (P=0.23). Of the 20 bats tested, only 15% attempted to feed on fruits treated with the synthetic blend, whereas no bats attempted to feed on untreated unripe fruits or on unripe fruits treated with pure pentane.

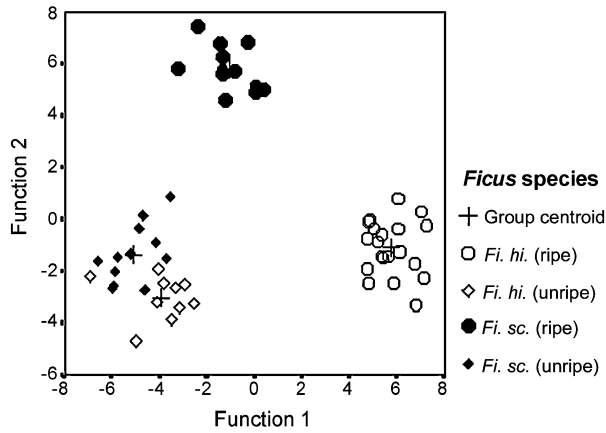


Fig. 5 Discriminant Function Analysis of the ripe and unripe fruit odors of *Ficus hispida* (*Fi. hi.*) and *Ficus scortechinii* (*Fi. sc.*). This analysis was based on the relative proportions of 15 compounds that commonly occurred in the headspace samples of both species (see Fig. 3 and Table 1). Both discriminant functions accounted for 98.1% of the total variance

Table 2 The relative effect of three cues (color, shape, and odor) on the foraging behavior of four species of pteropodid bat—*Balionycteris maculata* (*N*=1), *Chironax melanocephalus* (*N*=1), *Cynopterus brachyotis* (*N*=18), and *Cynopterus horsfieldi* (*N* =2)

Treatment	Sensory Cue	*N*	Frequency of Behavioral Response (%)			
			Circling	Approaching	Landing	Feeding
1) Ripe fruit	Color, shape, odor	22	100	100	100	100
2) Unripe fruit	Color, shape	20	85	70	15	5
3) Ripe fruit in black stocking	Shape, odor	20	100	100	95	95
4) Smeared fruit pulp	Odor	12	100	100	100	100

[a] *N* indicates the number of individual bats tested

Discussion

Fruit bats responded to both visual and chemical (olfactory) cues when foraging for figs. However, the strongest foraging reaction, resulting in a landing or feeding attempt, was almost exclusively associated with the presence of a ripe fruit odor, either in combination with visual cues, or when presented on its own. Thus, no visual cues were required for a fruit bat to locate the source of an odor. Fruit bats also used fruit odors to distinguish between ripe and unripe fruits. These results support the conclusions of previous studies (Acharya et al. 1998; Luft et al. 2003), that Old World fruit bats use mainly chemical, rather than visual, cues for the location and acquisition of food.

A total of 16 compounds were identified in the ripe fruit odor of *F. hispida*, and 13 in the ripe fruit odor of *F. scortechinii*—including alcohols, ketones, and esters. All 13 compounds that were identified in the ripe fruit odor of *F. scortechinii* were also present in the ripe fruit odor of *F. hispida*. This large overlap in fruit odor chemistry suggests that both species use the same compounds for bat attraction. However, none of the compounds identified in the fruit odors of either species is known to be associated with the attraction of bats (Bestmann et al. 1997; von Helversen et al. 2000; Knudsen and Tollsten 1995; Knudsen et al. 1993; Pettersson and Knudsen 2001; Pettersson et al. 2004). Indeed, many compounds that are commonly associated with Neotropical bat-pollinated flowers (e.g., sulfur-containing compounds) were completely absent (Bestmann et al. 1997; von Helversen et al. 2000).

Of the 16 compounds identified in the ripe fruit odors of figs, the methyl ketones and the secondary alcohols and their acetates are common fruit odor components within a wide

Table 3 Discrimination between ripe and unripe fruits (*Ficus hispida* and *Ficus scortechinii*) by four species of fruit bat (*Balionycteris maculata*, *Chironax melanocephalus*, *Cynopterus brachyotis*, and *Cynopterus horsfieldi*) in relation to fruit odor

Treatment	*N*[a]	Food Choice	Frequency of Feeding Attempts (%)
1) Unripe fruit vs. ripe fruit	20	Unripe fruit	0
		Ripe fruit	100
2) Unripe fruit vs. unripe fruit smeared with ripe fruit odor (from fruit pulp)	15	Unripe fruit	0
		Unripe fruit-smeared with ripe fruit odor (from fruit pulp)	93

[a] *N* indicates the number of individual bats tested

range of plant taxa (e.g., Laska 1990b; Jordan et al. 2001). In contrast, butyrates and hexanoates are much rarer. Interestingly, (*E*)-2-penten-3-yl esters, which were present in both species, have not, to the best of our knowledge, been reported from nature before. This suggests that a single, unique, bat-attracting compound could be present within the two fig species analyzed in this study. Alternatively, a combination of other compounds, particularly those that increase in production when fruits ripen (e.g., 2-heptanol and its acetate), could also function as attractants or markers, allowing the bats to differentiate between different fig species.

Both natural and synthetic fruit odors of *F. hispida* resulted in feeding attempts by fruit bats during bioassays. However, reaction rates to natural fruit odors were significantly higher than those displayed toward synthetic blends. Thus, the fruit bats could either detect subtle differences in chirality between the two treatments, or some additional compounds that are attractive to bats were missing from the synthetic blend. In the case of the former hypothesis, the use of racemic mixtures of 12 chiral compounds could at least partially explain the lower reaction rates of bats toward the synthetic blend used. Given the chiral nature of the receiving proteins, it seems likely that bats, and many other mammals, can distinguish between enantiomers of many different compounds (e.g., compounds 2, 3, 5, 7, 8, 9, 10, 12, 16, 17, 18, and 20, Table 1). Hence, the enantiomeric composition that occurs in nature could be of great importance to bat foraging behavior. However, the effect of enantiomeric composition on the foraging behavior of Old World fruit bats is currently unknown.

Spatial information on the precise location of fruits could also depend to some extent on the presence of low volatility compounds, such as sesquiterpenes (e.g., α-bergamotene), which were omitted from the synthetic blend used here. Compared to relatively high-volatility compounds, such as ketones and alcohols, sesquiterpenes are likely to produce a more localized plume of scent, which could allow bats to discriminate between ripe and unripe fruits situated in close proximity.

Further studies on the composition of fruit odors and the foraging behavior of fruit bats are needed to identify the key attractive compounds, and also to assess the occurrence of these compounds, more generally, within the genus *Ficus*. However, the results of this study have demonstrated the key importance of fruit odors for the mediation of interactions between bats and figs in the Paleotropics, and have also revealed many compounds of interest for future studies.

Acknowledgments We thank the Department of Wildlife and National Parks, Malaysia and the Economic Planning Unit of the Prime Minister's Department, for granting us permission to work in Malaysia. We also gratefully acknowledge Mr. Ahmad Bin Dagu, for assistance in the field, and two anonymous referees for comments on the manuscript. This project was funded by a grant from the Lubee Bat Conservancy to THK and RH and by funds from the German Science foundation (DFG) to EKVK and MA (KA 124 8-1).

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Introduction

Many herbivores are exposed to a diverse array of plant secondary compounds (PSC) naturally present in their diet. Many of these are recognized for their toxicity and generate physiological damage when consumed. The detoxification limitations hypothesis (Freeland and Janzen 1974) proposed that xenobiotic metabolism is the principal physiological mechanism responsible for neutralizing the harmful effects of PSC. This hypothesis suggested also that the paucity of mammalian dietary specialists is the result of constraints on the evolution of a xenobiotic metabolizing system capable of eliminating the large concentrations of similar toxins present in a specialist diet (Freeland and Janzen 1974; Marsh et al. 2006).

Previous studies on biotransformation mechanisms suggest that specialists differ intrinsically from generalists in enzymatic metabolism of PSC. Specialist herbivores appear to rely more on enzymes that add a functional group (Phase 1) and less on those that add an endogenous conjugate (Phase II) compared to generalist herbivores (McLean et al. 1993, 2001; McLean and Foley 1997; Boyle et al. 2001). In these studies, several species of marsupials that specialize on *Eucalyptus* had higher Phase I activity and lower Phase II, when exposed to *Eucalyptus* PSC compared to generalists (McLean and Foley 1997; Boyle et al. 1999, 2001). Additionally, *Eucalyptus* specialists expressed different levels of specific classes of Phase I enzymes (i.e., cytochrome P450s) than generalists exposed to the same compounds (Bolton and Ahokas 1997; Stupans et al. 1999).

We evaluated the prediction that a eutharian specialist woodrat (*Neotoma macrotis*) had higher levels of Phase I and lower levels of Phase II enzyme activities than a sympatric generalist woodrat (*N. lepida*). Both woodrats include oak (*Quercus* spp.) leaves in their diet. *Neotoma macrotis* is an oak specialist with up to 85% of its diet being *Quercus agrifolia* (Atsatt and Ingram 1983; Matocq 2002). In lab studies, populations of *N. macrotis* selected *Q. agrifolia* leaves over foliage from other dominant species (Atsatt and Ingram 1983). *Neotoma lepida* includes oak as a portion of their diet but is considered a generalist. In areas where *N. lepida* is sympatric with *N. macrotis*, the diet is primarily *Opuntia occidentalis*, cactus, and *Salvia apiana* (Cameron 1971; Atsatt and Ingram 1983). Oak is high in phenolics that are considered toxic to most other mammals (Atsatt and Ingram 1983; Lindroth and Batzli 1984; Thomas et al. 1988; Iason and Murray 1996). The differential consumption of oak by the specialist and generalist is predicted to be a result of xenobiotic metabolizing capabilities (Atsatt and Ingram 1983).

We took a comparative pharmacological approach to determine the xenobiotic metabolizing mechanisms used by the specialist and generalist when consuming oak foliage. We investigated the biotransformation activities of five major enzymes and three specific isozymes that include members from the two classes of xenobiotic metabolizing enzymes: Phase I and Phase II. The Phase I enzymes assayed were oxidation (cytochrome P450s; CYP) and reduction (NAD(P)H: quinone oxidoreductase; QOR). In addition to overall CYP concentration, catalyzed oxidations characteristic of three isozymes (CYP1A, CYP2B, and CYP3A) were included either for their role in biotransformation of PSC in a variety of animal systems and/or because they are common enzymes in pharmacological studies (Gregus et al. 1983; Hiroi et al. 1995; Parkinson 1996; Pass et al. 1999, 2001; Danielson 2002; Liukkonen-Anttila et al. 2003; Sivapathasundaram et al. 2003a). The Phase II enzymes assayed were UDP-glucuronosyltransferase (UGT), glutathione S-transferase (GST), and sulfotransferase (SULT), each chosen for their common role in the biotransformation of a variety of compounds (Gregus et al. 1983; Sivapathasundaram et al. 2003b). UGT and GST represent the major Phase II enzymes in mammals (Dutton 1980; Daniel 1993). SULT is an important alternative to glucuronidation for phenolics (Parkinson 1996).

Materials and Methods

Study System *Neotoma macrotis* and *N. lepida* were trapped at Caspers Wilderness Park in Orange County, CA, USA. The animals were transported to the University of Utah Animal Facility, housed in individual cages (48×27×20 cm) with bedding and cotton batting and put on a 12–12 hr L/D cycle for at least 3 months before experiments to allow for Sin Nombre hantavirus screening. All the animals were maintained on standard rabbit chow (Harland Teklad formula 2031) and water ad libitum before experimentation. All procedures were approved by the University of Utah IACUC.

Dietary Treatments The animals were fed one of two diets (control or 70% oak). Control diet consisted of the same rabbit chow used for the maintenance diet with the exception that it was in powdered form. *Quercus agrifolia* used in diet treatments was stripped from several trees (>10) at sites where animals were trapped in California and stored at −20°C until use. Foliage was homogenized by grinding in a Waring blender (model CB-5) with dry ice until it passed through a 1-mm screen. Homogenization of the oak was necessary to control for oak diet intake but may have caused some chemical oxidation of the PSC present in the foliage. Oak diets were prepared by adding ground oak to ground rabbit chow on a percent dry weight basis of each component. The diets that contained oak were prepared daily to minimize the potential for chemical degradation. Total food intake (g dry matter) was measured each day. For the control treatment, the specialist, *N. macrotis* (N=7), and generalist, *N. lepida* (N=7), were fed the control diet of rabbit chow for 5 days to determine constitutive levels of xenobiotic metabolizing enzymes. Animals in another group of specialists (N=9) and generalists (N=7) that were given the 70% oak treatment diet were first acclimated to a 25% oak diet for 3 days, followed by 50% oak diet for 1 days, and then fed 70% oak diet for 3 days. Before treatment, all the animals were given a control diet that consisted of standard rabbit chow (Harland Teklad) for several weeks.

Microsomal Preparation and Enzyme Assays Enzyme activity assays were used to measure activity of hepatic enzymes among species and treatment groups. Enzyme activities were determined in subcellular fractions isolated from whole liver tissue. On the last day of each dietary treatment, the animals were killed with CO_2. The liver was immediately perfused in situ by injecting cold isotonic saline into the hepatic portal vein, and the gall bladder was removed. The liver was then removed and weighed. Microsomal and cytosolic fractions were prepared by differential ultracentrifugation as described by Franklin and Estabrook (1971) and stored at −80°C until assayed for activity. Protein concentrations were determined colorimetrically by the method of Lowry (1951) to standardize enzyme activity to protein concentration.

Cytosolic NAD(P)H/quinone oxidoreductase (QOR) activities were determined by the dicoumarol-inhibited rate of reduction of 2, 6-dichlorophenolindophenol (Aldrich Chemical Co., Milwaukee, WI, USA) by NADH at pH 7.4 detected with spectrophotometry (Benson et al. 1980). Overall CYP concentration was determined from microsomes by using the carbon monoxide difference spectrum on a spectrophotometer by the method of Omura and Sato (1964) that measures the reduced P450/CO complex characteristic of all CYP isozymes cumulatively. CYP1A activities were determined by the method of Klotz et al. (1984) that measures the rate of resorufin production during the metabolism (*O*-deethylation) of 7-ethoxyresorufin (EROD; Sigma Chemical Co., St. Louis, MO, USA). CYP2B activities were determined according to Lubet et al. (1985) by measuring the rate of

resorufin production during the metabolism (de-alkylation) of pentoxyresorufin (PROD; Sigma). CYP3A activities were determined by testosterone 6$\tilde{\beta}$ hydroxylation (Sigma) where the testosterone metabolite (6$\tilde{\beta}$ hydroxytestosterone) was separated by high-performance liquid chromatography and quantified from its absorbance at 236 nm (Guengerich et al. 1986).

Hepatic microsomal UGT activities were determined by spectrophotometry (Bock et al. 1983) in reactions containing detergent (0.05% Triton X-100; Sigma). We measured the UDPGA-dependent disappearance rate of *p*-nitrophenol (Sigma). Cytosolic GST activities were determined by spectrophotometry (Habig and Jakoby 1981) detecting change in absorbance at 340 nm upon conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB, a general reference substrate; Aldrich Chemical Co., Milwaukee, WI, USA). SULT cytosolic activities were determined by detecting the PAPS-dependent disappearance rate of *p*-nitrophenol (Sigma) with spectrophotometry (Sekura et al. 1981).

The probe substrates used in the assays are considered isozyme specific. For example, isozyme CYP 1A catalyzes the metabolism of 7-ethoxyresorufin; SULT enzymes catalyze the metabolism of *p*-nitrophenol. Although these protocols were designed and validated in laboratory rats, they are often used in disparate species. Given that laboratory rats are in the same suborder (Myomorpha) as woodrats, the use of these assays is likely to provide a valid estimate of enzyme specific activities. The probe substrates, CDNB and *p*-nitrophenol, are relatively nonspecific substrates for GST and UGT isozymes, respectively. However, CDNB generally targets the alpha class of GST in liver assays and *p*-nitrophenol targets UGT1A's. The SULT substrate targets SULT1 that is used for phenol metabolism.

Statistical Analysis Body mass and the activities of each detoxification enzyme analyzed were compared by using a two-way analysis of variance (ANOVA) with species and diet as the independent variables. When significant ($P<0.05$), a post hoc Fisher's LSD for pair-wise comparisons was conducted. Because there were differences in body mass, an ANCOVA was used to compare dry matter intake and liver mass by using body mass as a covariate, and diet and species as the independent factors. Systat 10 (SPSS, Chicago, IL, USA) was used for analysis.

Results

Body Mass, Dry Matter Intake, and Liver Mass The body mass of the specialist was approximately 20% greater than the generalist (Table 1). As a result of the difference, food intake was analyzed with body mass as a covariate. Dry matter intake did not differ between

Table 1 Mean body mass, dry matter intake (DMI) and liver mass from the specialist, *N. macrotis* and the generalist, *N. lepida* fed control diets or diets containing 70% oak (*Quercus agrifolia*; data represented as means±1 SE)

Variable	*N. macrotis* (specialist)		*N. lepida* (generalist)	
	Control diet	Oak diet	Control diet	Oak diet
Body mass (g)	208±15.4^{a}	204±13.6^{a}	161±15.4^{b}	162±15.4^{b}
Dry matter intake (g)	10.5±1.5^{a}	24.7±1.3^{b}	11.5±1.5^{a}	22.5±1.5^{b}
Liver mass (g)	5.05±0.36^{a}	4.15±0.32^{b}	5.59±0.36^{a}	4.94±0.36^{b}

^{a,b} Denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD

the specialist and generalist (Table 2). However, both species had twofold higher intake when consuming oak diet compared to control diet (Table 1). Liver mass was not different between the specialist and generalist, but liver mass was significantly smaller in animals on the oak treatment compared to control groups in both species irrespective of body size (Table 2).

Cytochrome P450 Concentration Overall, there were no significant cytochrome P450 concentration differences among the treatment groups (Fig. 1). However, there was a trend for the generalist, *N. lepida*, to have higher activity than the oak specialist (Table 3).

Quinone Oxidoreductase The specialist had higher QOR activity than the generalist, *N. lepida* (Fig. 1). Specifically, the levels of constitutive QOR activity were more than two times higher in the specialist compared to the generalist and did not change on oak diet (Table 3).

Cytochrome P450 Isozymes Activity levels for the specific isozymes CYP1A, CYP2B, and CYP3A varied among the groups (Fig. 2). CYP1A activities in the generalist were constitutively 30% higher than that of the specialist (Fig. 2). Neither the specialist nor generalist significantly increased CYP1A activity between control and oak diet (Table 3). CYP2B activities in the control treatment groups were similar (Fig. 2). However, there was a significant effect of diet on CYP2B activities (Table 3). Both the specialist and generalist increased activity by more than 20% on the oak treatment. Finally, CYP3A activity levels were not different between the specialist and generalist fed control or oak diets (Table 3).

UDP-Glucuronosyltransferases Levels of UGT activity were constitutively different between the specialist and generalist (Fig. 3). UGT activity in the specialist was nearly two times higher compared to the generalist. Levels of UGT did not change with diet treatment groups and remained higher in the oak specialist compared to the generalist (Table 3).

Sulfotransferase Levels of SULT activity were constitutively different between the specialist and generalist (Fig. 3). SULT activity in the generalist was more than two times higher than the specialist in both treatments (Table 3). There was no change in SULT activity by either the specialist or the generalist from control to oak diets.

Table 2 Summary of *F*-values, degrees of freedom (*df*) and *P* values for ANOVA analysis of body mass and ANCOVA analyses of food intake and liver mass among *N. macrotis* and *N. lepida* in two treatment groups: control diet and 70% oak diets

ANOVA/ANCOVA			
Source of variation	*F*	*df*	*P*
Body mass			
Species	8.555	1,26	0.007
Diet	0.006	1,26	0.939
Species X diet	0.043	1,26	0.836
Dry matter intake			
Species	0.137	1,25	0.714
Diet	78.10	1,25	<0.001
Species X diet	1.281	1,25	0.268
Covariate (body mass)	0.070	1,25	0.793
Liver mass			
Species	2.918	1,25	0.100
Diet	5.237	1,25	0.031
Species X diet	0.136	1,25	0.716
Covariate (body mass)	31.89	1,25	<0.001

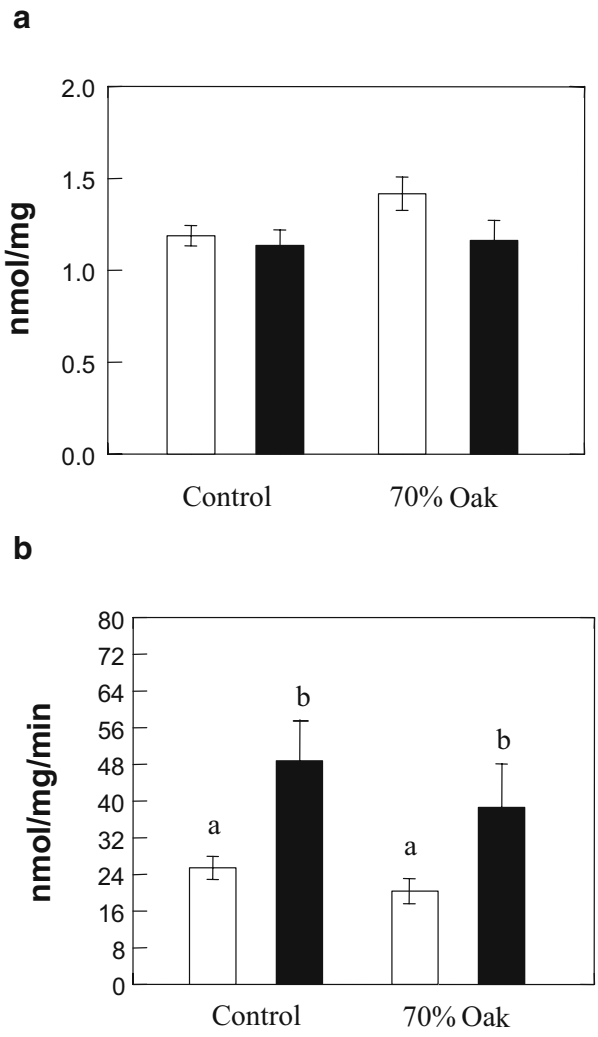


Fig. 1 Hepatic Phase I enzymes (mean±SE) in the specialist, *N. macrotis* (*filled bar*) and the generalist, *N. lepida* (*open bar*) fed control diets or diets containing 70% oak (*Quercus agrifolia*): **a** Cytochrome P450 Concentration; **b** NAD(P)H/quinone oxidoreductase (QOR) activity. Data are represented as bars and different letters (*A* and *B*) denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD

Glutathione Transferase Levels of constitutive GST activity were significantly higher in the specialist compared to the generalist (Table 3). There was a change in GST activity in the generalist when consuming the oak diet treatment (Fig. 3). The generalist significantly decreased GST activity by approximately 25% from the control diet compared to the oak diet treatment group (Table 3).

Discussion

Previous research suggests that mammalian xenobiotic metabolism is the primary physiological mechanism that permits specialist feeders to consume high levels of a limited class of plant secondary compounds (PSC). We predicted that the mammalian oak specialist would have higher activities of Phase I xenobiotic metabolizing enzymes and

Table 3 Summary of *F* values, degrees of freedom (*df*) and *P* values for ANOVA analysis of xenobiotic metabolizing enzyme assays among *N. macrotis* and *N. lepida* in two treatment groups: control diet and 70% oak diets

ANOVA			
Source of variation	*F*	*df*	*P*
QOR			
Species	18.90	1,26	<0.001
Diet	0.777	1,26	0.386
Species X diet	0.059	1,26	0.810
Cytochrome P450 concentration			
Species	3.46	1,26	0.074
Diet	2.39	1,26	0.134
Species X diet	1.515	1,26	0.229
CYP1A (EROD)			
Species	10.006	1,26	0.004
Diet	1.184	1,26	0.287
Species X diet	0.824	1,26	0.372
CYP2B (PROD)			
Species	1.462	1,26	0.237
Diet	15.02	1,26	0.001
Species X diet	0.023	1,26	0.881
CYP3A			
Species	0.431	1,26	0.517
Diet	0.469	1,26	0.500
Species X diet	1.456	1,26	0.238
UGT			
Species	74.95	1,26	<0.001
Diet	0.365	1,26	0.551
Species X diet	0.681	1,26	0.417
SULT			
Species	19.78	1,26	<0.001
Diet	1.594	1,26	0.218
Species X diet	1.783	1,26	0.193
GST			
Species	72.23	1,26	<0.001
Diet	6.247	1,26	0.019
Species X diet	1.057	1,26	0.313

lower activities of Phase II enzymes than the generalist. However, both the specialist and generalist had high levels of different Phase I enzyme activities. Furthermore, the specialist had higher overall levels of Phase II enzyme activities than the generalist in two of the three Phase II enzymes assayed. There were few differences in enzyme activity levels from control diet to an oak diet. There was only one enzyme induced by oak diet, CYP2B, and it by little more than 20% in both specialist and generalist. In addition, the generalist appeared to suppress GST activities by approximately 30% on an oak diet, although there was not a significant interaction effect. There were major differences in constitutive enzyme activity between the species. The specialist, *N. macrotis*, had higher activity of QOR, UGT, and GST. QOR and UGT activities were both twofold higher, and GST activities were 37% higher than the generalist when both were consuming control diet. When both species were consuming an oak diet, GST activities were twofold higher in the specialist than the generalist. The generalist, *N. lepida*, had higher constitutive activity levels of CYP1A and SULT. CYP1A activities were approximately 30% higher, and SULT activities nearly threefold higher than the specialist.

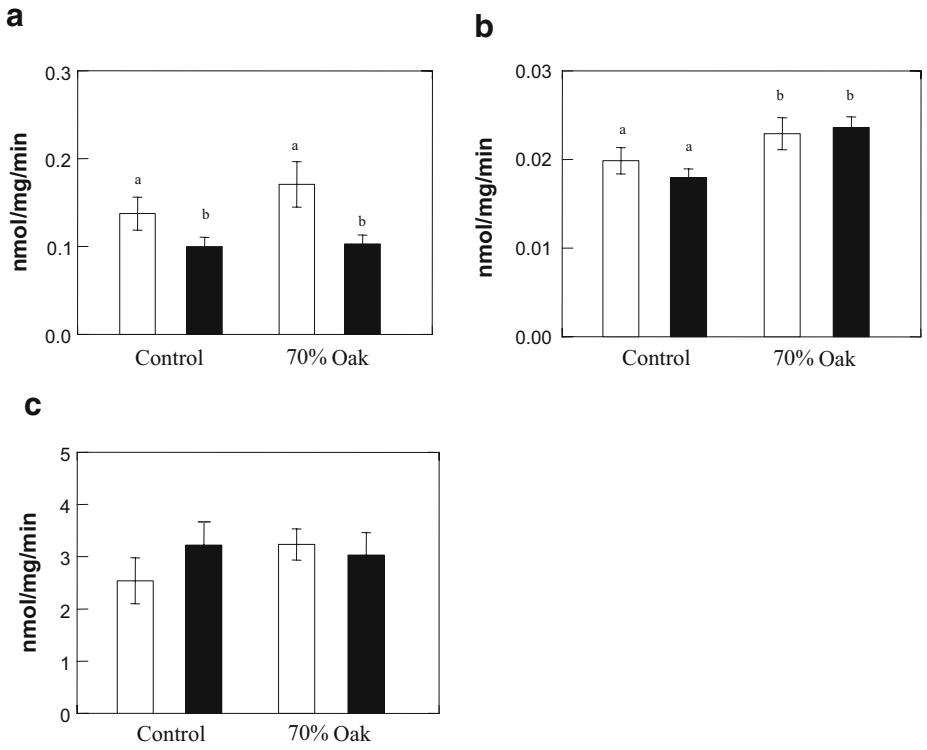


Fig. 2 Hepatic cytochrome P450 isozyme activities (mean±SE) in the specialist, *N. macrotis* (*filled bar*), and the generalist, *N. lepida* (*open bar*), fed control diets and diets containing 70% oak (*Quercus agrifolia)*: **a** Isozyme CYP1A; **b** Isozyme CYP2B; **c** Isozyme CYP3A. Data are represented as the mean+SE. Bars with different letters (*A* and *B*) denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD

Oak expresses high levels of phenolic PSC (Atsatt and Ingram 1983). Phenolics, including tannins, are known to be deterrent or toxic to herbivores. Their toxic effects have been demonstrated in a number of plant–herbivore interactions, and protective mechanisms are required to allow for their consumption (Feeny 1970; Haukioja et al. 1985; Rhoades 1985; Rossiter et al. 1988). Tannins may act as digestability reducers and bind dietary protein during digestion, resulting in elevated levels of fecal nitrogen. Since both species have similar nitrogen retention efficiencies, the digestability reducing effect of tannins and the level of protection provided by proline-rich salivary proteins is also likely similar (Atsatt and Ingram 1983). Tannins are only a portion of the total polyphenols that have toxic potential in oak. Furthermore, their effect is not limited to digestability reduction because they can be degraded and absorbed into circulation resulting in toxicity. In either case, xenobiotic metabolizing enzymes may either enhance or reduce the harmful effects of oak phenolics (Foley and McArthur 1994).

The fate of most absorbed phenolics involves biotransformation by Phase I oxidation (most notably carried out by cytochrome P450s). CYP1A was 30% higher in the generalist compared to the oak specialist, but activity was not induced with oak diet. A number of plant compounds including caffeine, indole-3-carbinol, and warfarin are metabolized by CYP1A (Ayalogu et al. 1995; Vang et al. 1999; Kuo et al. 2006). CYP2B was induced by around 20% in both the specialist and the generalist. Elevation of this enzyme on an oak

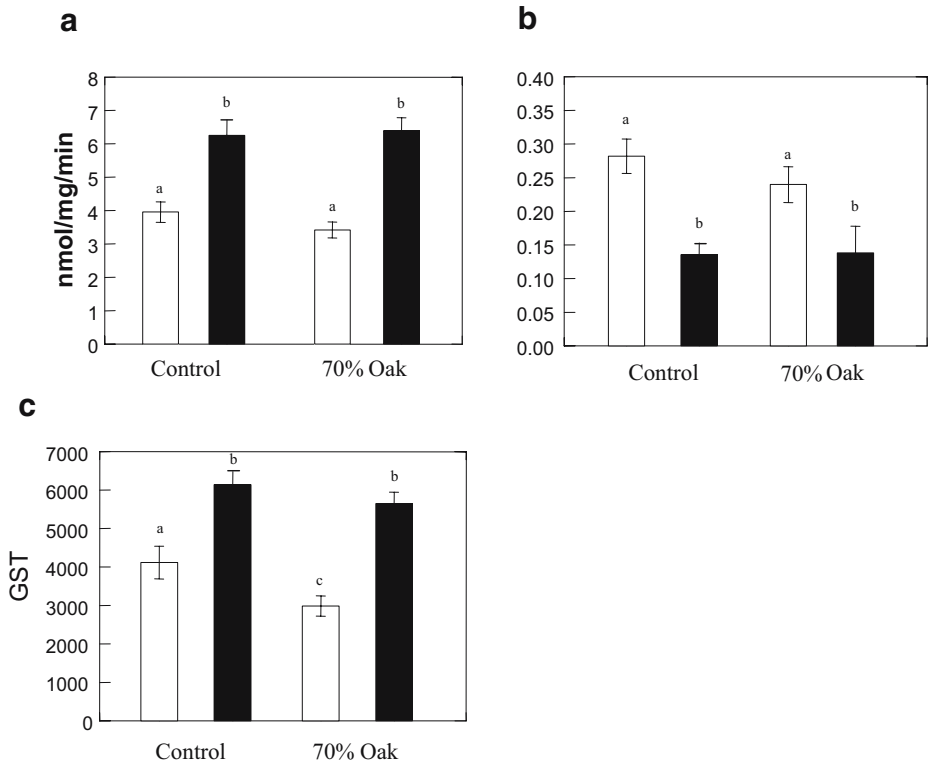


Fig. 3 Hepatic conjugation enzyme activities (mean±SE) in the specialist, *N. macrotis* (*filled bar*), and the generalist, *N. lepida* (*open bar*), fed control diets and diets containing 70% oak (*Quercus agrifolia*): **a** UDP-glucuronosyltransferase (UGT); **b** Sulfotransferase (SULT); **c** Glutathione transferase (GST). Data are represented as the mean+SE. Bars with different letters (*A*, *B* and *C*) denote means that are significantly different ($P<0.05$) as determined by Fisher's LSD

diet rich in polyphenolics is somewhat surprising because a number of plant phenolics (e.g., tannic, ellagic, and protocatechuic acids) are known to inhibit the activity of CYP2B (Szaefer et al. 2003).

The induction of CYP2B by both the specialist and generalist indicates that both have similar capacities to induce this enzyme when consuming an oak diet. In a field study, the specialist had a higher rate of CYP2B activity, as measured by in vivo hexobarbital clearance rates, than the generalist (Dearing et al. 2006). The enhanced ability of the specialist to metabolize hexobarbital under field conditions is not surprising due to the greater consumption of oak in the wild. Therefore, while the generalist consumes mostly *Opuntia occidentalis*, cactus, and *Salvia apiana* in its natural habitat, it would have lower levels of CYP2B activity. However, the activities observed here indicate that the generalist has the capacity, when consuming oak, to increase CYP2B activity to an extent similar to the oak specialist.

The differences in the pattern of conjugation enzymes used by the two species could explain their natural diet preferences. The oak specialist had higher constitutive activity levels of UGT, whereas the generalist had higher levels of SULT. Species variation in the rates of glucuronidation (UGT) and sulfation (SULT) can lead to differences in tolerance for phenolic compounds because there are differences in capacity for conjugation between these two Phase II enzymes (Parkinson 1996). SULT requires PAPS (3′-phosphoadenosine-

5′-phosphosulfate) as a sulfate donor to conjugate xenobiotics, and PAPS is in limited supply. Thus, SULT activity in vivo has a relatively low capacity. UGT's cofactor, UDP-glucuronic acid, is not as limited as PAPS, and UGT has a higher capacity as a result (Klaassen and Boles 1997). This difference may be more profound when oak is a constant diet component. The cost or toxic effect of chronic intake of a particular compound can be different from that produced by short-term exposure, particularly with regard to cofactor abundance. In a diet containing high levels of phenolics that are consumed chronically, the difference may alter a species' ability to specialize.

While the constitutive activity differences may explain oak tolerance, any inference regarding these differences should be viewed cautiously, as they were not altered by a change from control to oak diet. Many of the differences measured between the specialist and generalist were constitutive. These may be due to species phylogenetic distance and not necessarily short-term dietary behavior. The fact that few of the enzymes analyzed were affected by the change from control diet to oak diet constricts inference concerning which enzymes metabolize the PSC in oak. Many xenobiotic metabolizing enzymes are also important in the metabolism of endogenous compounds (e.g., hormones, bilirubin, and bile acids; Parkinson 1996). Therefore, while differences in xenobiotic enzymes likely influence oak consumption, assays to look specifically at oak phenolic metabolism as influenced by these specific enzymes will be necessary. The reduced size of the liver in both the specialist and generalist may indicate that mechanisms other than hepatic xenobiotic metabolizing enzymes are involved. For example, some intestinal proteins (multi-drug resistant proteins) prevent the absorption of compounds into circulation (Bodo et al. 2003).

Although a theory has emerged may postulate that specialists rely more heavily on Phase I than Phase II xenobiotic metabolizing enzymes in comparison to generalists, this research indicates that it is not a widespread pattern broadly applied to all specialist/generalist comparisons. Although this study included many of the major xenobiotic metabolizing enzymes used by mammals, and although we found differences between the specialist and generalist species, future work with inhibitors and cloning of specific genes may be necessary to tease apart the role these enzymes play.

Acknowledgement We thank committee members Dr. G. Yost, Dr. F. Goller, Dr. D. Feener for their invaluable input. We also thank Dr. J. Sorensen, Dr. M. Skopec and Ann-Marie Torregrossa for critical reading of the manuscript and the undergraduates in Dr. Dearing's lab, especially Katie Young and John Matthews, for assistance with feeding, tissue collection as well as collection and maintenance of woodrats. Research was supported by National Science Foundation (NSF IBN 023402).

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Introduction

It is well-known that secondary metabolites (SMs) are synthesized uniquely in specific plant species. Various viewpoints have served as a basis for the investigation of their function in nature: attraction of pollinators (Pellmyr and Thien 1986), host plant selection of phytophagous insects (Hsiao 1985; Endo et al. 2004), and interference effects between plants (Harper 1977; Rice 1984). The antimicrobial activities of SM have also been investigated (Osbourn 1996), and the active compounds behind the activities of some have been identified and used as pesticides either in their natural or synthetic forms.

Many studies have also reported about the antiphytopathogenic fungal activities of essential oils in aromatic plants, including spices and herbs (Muller-Riebau et al. 1995; Letessier et al. 2001; Oxenham et al. 2005). While the antifungal effects of volatile compounds of plants, for example the 2-hexenal "wound volatile" compound, have been investigated extensively (Hamilton-Kemp et al. 1992; Archbold et al. 1997), antimicrobial investigation of aromatic plants has been limited, despite the high degree of activity shown by these plants against phytopathogens (Shimoni et al. 1993; Soylu et al. 2006).

The use of volatiles from plants as bio-fumigation materials to reduce soil-borne diseases also has been reported. Indian mustard and hairy vetch, which releases antifungal compounds, allyl isothiocyanate and cyanamide, respectively, are possible controls against *Fusarium* wilt of spinach (Takehara et al. 2004).

Many SMs are phytotoxic. Several methods are known for studying phytotoxic effects of volatile compounds (Reuveni et al. 1984; Hamilton-Kemp et al. 1992; Kong et al. 1999). Fujii et al. (2005) developed the "dish pack method" to study effects on seed germination by using six-well multidishes. The speed of diffusion and intensity of activity are estimated based on the relationship of the distance of the seeds from the test plants or chemicals and the growth inhibition of the seed. We applied the method to check the anti-phytopathogenic fungal activities of volatile compounds from 52 samples of spices and herbs against the soil-borne phytopathogen, *Fusarium oxysporum*. Our objective was to determine the component of the species with the strongest anti-fungal effect.

Materials and Methods

Screening of Spices and Herbs

Dried samples of 52 spices and herbs were tested (Table 1): 37 (gifts from YASUMA, Tokyo, Japan) and 15 others (cultivated in the fields of Miyagi Prefectural Agriculture and Horticulture Research Center, National Institute for Agro-Environment Science, or Ferdowsi University, Iran). Samples were dried in a hot air circulation oven at 60°C for 1–2 days and then ground finely with a Japanese traditional grinder "Yagen" just before the experiment.

The antifungal bioassay was conducted following the principles of the dish pack method (Fujii et al., 2005), using six-well multidishes (Nunc, external dimensions: 128×86 mm, 35 mm-diameter wells; Fig. 1). Ground sample (500 mg) was placed at the lower left, 0-mm-distance well (source well) of the multidish, while 2.5 ml of potato dextrose agar (PDA) with streptomycin (200 ppm) was poured into each of the other five wells and then allowed to harden. A 4-mm plug of a mycelial disk of *F. oxysporum*, cultured on PDA for 5 days at 25°C, was placed on top of the agar, with the side of mycelial growth facing down. *F. oxysporum* was obtained originally from the crown of a diseased strawberry, and the cultures were established and maintained on PDA. The multidish was covered, and the

Table 1 Mycelial growth inhibitions of 52 species of spices and herbs against *Fusarium oxysporum*

Common name (part used)[a]	Donor spice and herb		Mycelial growth
	Scientific name	Family	inhibition (%)[c]
Garlic[b]	*Allium sativum*	Liliaceae	0
Scallion (root)	*Allium fistuiosum*	Liliaceae	13
Onion[b]	*Allium cepa*	Liliaceae	0
Scallion (leaf and stem)	*Allium fistuiosum*	Liliaceae	0
Chinese pepper[b]	*Zanthoxylum bungeanum*	Rutaceae	8
Chinpi[b]	*Citrus unshiu*	Rutaceae	−5
Clove[b]	*Eugenia aromatica*	Myrtaceae	31
Allspice[b]	*Pimenta officinalis*	Myrtaceae	4
Juniper berry[b]	*Juniperus communis*	Cupressaceae	25
Mace[b]	*Myristica fragrans*	Myristicaceae	13
Nutmeg[b]	*Myristica fragrans*	Myristicaceae	4
Red pepper[b]	*Capsicum annuum*	Solanaceae	31
Houttuynia (root)	*Houttuynia cordata*	Saururaceae	6
Houttuynia (leaf and stem)	*Houttuynia cordata*	Saururaceae	25
Caraway[b]	*Carum carvi*	Umbelliferae	22
Dill (seed)[b]	*Anethum graveolens*	Umbelliferae	16
Black zira	*Bunium persicum*	Umbelliferae	63
Coriander[b]	*Coriandrum sativum*	Umbelliferae	10
Fennel (seed)[b]	*Foeniculum vulgare*	Umbelliferae	0
Parsley[b]	*Petroselium sativum*	Umbelliferae	13
Celery (seed)[b]	*Apium graveolens*	Umbelliferae	6
Seri (root)	*Oenanthe javanica*	Umbelliferae	16
Cumin[b]	*Cuminum cyminum*	Umbelliferae	41
Dill (leaf and stem)	*Anethum graveolens*	Umbelliferae	9
Seri (leaf and stem)	*Oenanthe javanica*	Umbelliferae	13
Anise[b]	*Pinpinella anisum*	Umbelliferae	6
Ajowan[b]	*Trachyspermum ammi*	Umbelliferae	5
Cardamom[b]	*Elettaria cardamomum*	Zingiveraceae	38
Ginger[b]	*Zingiver officinale*	Zingiveraceae	9
Brown cardamom[b]	*Elettaria cardamomum*	Zingiveraceae	31
Turmeric[b]	*Curcuma domestica*	Zingiveraceae	3
Rosemary[b]	*Rosemarinus officinalis*	Labiatae	21
Sage[b]	*Salvia officinalis*	Labiatae	0
Thyme[b]	*Thymus vulgaris*	Labiatae	15
Ravender (leaf and stem)	*Lavandula augustifolia*	Labiatae	6
Ravender (root)	*Lavandula augustifolia*	Labiatae	13
Savory[b]	*Satureja hortensis*	Labiatae	16
Basil[b]	*Ocimum basilicum*	Labiatae	14
Origan[b]	*Origanum vulgare*	Labiatae	16
Shiso (green)	*Perilla frutescens*	Labiatae	14
Shiso (red)	*Perilla frutescens*	Labiatae	21
White pepper[b]	*Piper nigricum*	Piperaceae	13
Black pepper[b]	*Piper nigricum*	Piperaceae	3
Laurel[b]	*Laurus nobilis*	Lauraceae	0
Cassis[b]	*Cinnmomum cassia*	Lauraceae	0
Cinnamon[b]	*Cinnamomum verum*	Lauraceae	8
Tarragon[b]	*Artemisia dracunculus*	Compositae	0
Chamomile (leaf and stem)	*Anthemis nobilis*	Compositae	16
Chamomile (root)	*Anthemis nobilis*	Compositae	6

Table 1 (continued)

Common name (part used)[a]	Donor spice and herb		Mycelial growth
	Scientific name	Family	inhibition (%)[c]
Chamomile (flower)	*Anthemis nobilis*	Compositae	13
Pepper tree[b]	*Schinus molle*	Anacardiaceae	−5
Safran crocus[b]	*Crocus sativus*	Iridaceae	10

[a] For spices and herbs without specified part(s) the edible parts were used.
[b] These spices and herbs were gifts from YASUMA, and the rest were cultivated at our research sites.
[c] Mycelial growth inhibitions shown are the average of the two 41-mm wells. Black zira, cumin, and cardamon had three replicates each, while other values were derived from a single experiment. Negative values indicate stimulated mycelial growth.

sides were sealed with adhesive tape to prevent compound losses because of volatilization. A 1-mm headspace between the multidish cover and the wells allowed volatiles from the samples to spread throughout the multidish. The degrees of vapor diffusion and antifungal activity were estimated by the relationship of the degree of fungal inhibition of the mycelial disk and its distance from the source well. The multidish was incubated in a dark growth chamber for 3 days at 25°C, and then the growth of the mycelial colony in each well was determined by measuring its increase in diameter from that of its original 4 mm diameter. A

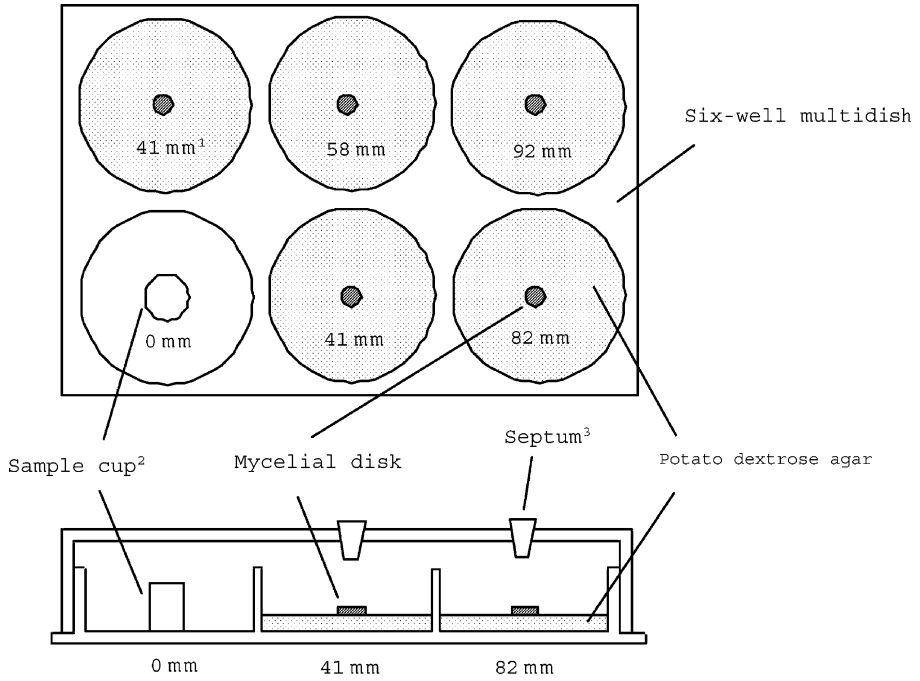


Fig. 1 Top and side views of the dish pack method used for testing the antifungal activities of volatile compounds. *1*, The distance from the sample or compound. *2*, Source well or cup (in the case of authentic compounds). *3*, Septa were attached for headspace vapor sampling done through the use of a Hamilton gas-tight syringe and analyzed by GC-MS analysis

control dish was set up in the same manner, except that the source well contained no sample. Finally, the mycelial growth inhibition was calculated as follows:

$$\textit{Mycelial growth inhibition}(\%) = \begin{pmatrix} \textit{growth of the control mycelial colony} \\ -\textit{growth of the treatment mycelial colony} \end{pmatrix} \Big/ (\textit{growth of the control mycelial}) \times 100$$

No replications were made during the first bioassay, but two additional replicates were done for black zira, cumin, and cardamom after initial results showed their high antimycelial activities.

Isolation of Antifungal Volatiles from Black Zira and Their Antifungal Activities

The presence of volatiles from black zira was analyzed with the headspace method. A finely ground sample of black zira (200 mg) was put into a glass vial equipped with an aluminum crimp seal cap with a polytetrafluoroethylene/silicone septum and was kept for 30 min under laboratory conditions. A headspace vapor sample (1 ml) was withdrawn from the bottle through the septum with a Hamilton gas-tight syringe and then immediately injected into the gas chromatography–mass spectrometry (GC-MS). Spectra were recorded with a QP-5050A instrument (SHIMAZU, Kyoto, Japan) using an EQUITY-5 column (Supelco, 30 m×0.25 mm, inside diameter 0.25 μm). Each compound was identified by comparing its mass spectrum in the National Institute of Standards and Technology library and with the retention time of an authentic sample on the GC. The quantity of each compound was determined by comparing the peak area of the compound with that of its authentic sample. The operating conditions of GC-MS were as follows: inlet, 200°C; column oven, 40°C for 30 s, then programmed to rise at 8°C/min to 160°C, which was maintained for 5 min. Splitless injection into the GC-MS with 1.0 ml of vapor phase sample or 2.0 μl of the liquid sample was applied. The concentrations of volatile compounds from black zira were calculated from their peaks. These headspace samplings and analyses were not replicated.

The corresponding authentic samples of the seven detected compounds were also tested for their activities against *F. oxysporum* by applying the same procedure used in testing the dried samples, except that a cup containing 50 μl of the authentic compound was placed into the source well instead of the dried samples (Fig. 1). Based on previous tests (Fujii, unpublished report), this amount was found as the most appropriate level for tested compounds to show signs of activity. Authentic samples of α-pinene, β-pinene, limonene, cuminaldehyde, and γ-terpinene were purchased from Wako Pure Chemicals, *p*-cymene from Sigma Chemical, and myrcene from Tokyo Chemical Industry. Four replications were made for the bioassay. To measure the vapor concentration of the authentic compound in each well, another five multidishes were set out in the same manner as above, but without mycelial disks. The five multidishes represented each of the five well distances, 0, 41, 58, 82, and 92 mm, and the collection of vapor from the assigned dish for each well was done just once to prevent unusual diffusion of vapor during the withdrawal process. The vapor was collected 24 h after the test compound was placed into the multidish by using a Hamilton gas-tight syringe (1.0 ml) through a septum located on top of each well. Headspace sampling and analysis of each well were not replicated. The same procedures, as described previously, were followed for the GC-MS analysis and the calculation of mycelial growth inhibitions of the authentic compounds. Cuminaldehyde and *p*-cymene showed the highest antifungal activities and were further

investigated against another soil-borne phytopathogenic fungus, *Varticillium dahliae*, and foliar phytopathogenic fungi, *Botrytis cinerea* and *Alternaria mali*, which were obtained from diseased chrysanthemum, strawberry fruit, and apple leaf, respectively. The fungi were precultured on PDA as for *F. oxysporum*, except that their preculture periods were different: 10 days for *V. dahliae*, 3 days for *B. cinerea*, and 5 days for *A. mali*. The testing periods were 2 days for *B. cinerea* and 3 days for *V. dahliae* and *A. mali*.

The biological activities of cuminaldehyde and *p*-cymene were also evaluated based on their specific activity, expressed as (EC_{50}), which is the effective concentration of the compound to induce half-maximum inhibition. In our study, the EC_{50} values of *p*-cymene and cuminaldehyde against *F.oxysporum* were calculated with probit analysis (Finney 1971), and then the total activities were calculated as follows: Total activity = $(1/EC_{50}) \times$ concentration in Black zira (μg/g plant).

Results

Mycelial growth inhibitions of 52 spices and herbs against *F. oxysporum*, based on the average result of the two 41-mm wells, are shown in Table 1. Forty-two samples showed varying degrees of mycelial growth inhibition, with black zira the strongest, followed by cumin and cardamom. Mycelial growth inhibition of these three samples at each well was evaluated (Fig. 2). Black zira showed a significantly strong mycelial growth inhibition over cumin and cardamom at the 41-mm wells. Thus, further investigation to determine the main component(s) responsible for the antifungal activity was done.

Seven volatile compounds, γ-terpinene, limonene, *p*-cymene, β-pinene, α-pinene, cuminaldehyde, and myrcene, were detected from black zira from headspace sampling and GC-MS analysis (Table 2). All these are terpenes, except for cuminaldehyde. To confirm which compounds were antifungal, authentic samples of the seven identified compounds were investigated for mycelial growth inhibition against *F. oxysporum.* Cuminaldehyde showed the highest inhibition at 92% in the 41-mm wells (Table 3), declining sharply to 18% in the farthest well (92 mm). *p*-Cymene followed at 56% (41-mm wells) with a gradual decline that reached 48% in the farthest well.

Vapor phase concentrations of the six terpenes in the 41-mm wells were each more than 100 ppm, while that of cuminaldehyde was 0.147 ppm, indicating the higher inhibitory

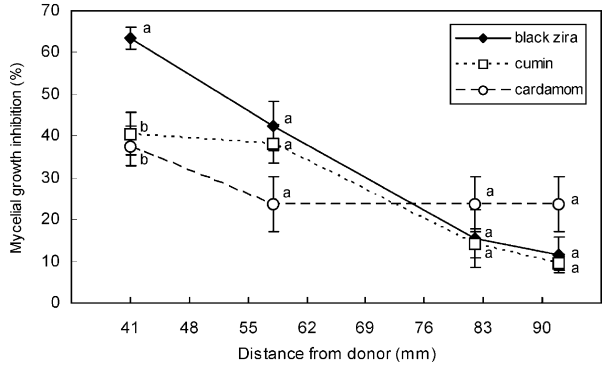


Fig. 2 Relationship between well distances from samples (black zira, cumin, and cardamom) and mycelial growth inhibition of *Fusarium oxysporum*. Each value represents the mean of three replicates. *Bars* represent standard error of the mean. The same letters at each distance indicate that the values are not significantly different ($P<0.05$) according to Tukey's test

Table 2 Volatile compounds from black zira by headspace sampling and GC-MS analysis

Compound	Concentration (μg/g plant)[a]
γ-Terpinene	14.4
Limonene	4.42
p-Cymene	4.31
β-Pinene	2.94
α-Pinene	2.37
Cuminaldehyde	2.23
Myrcene	1.01

[a] Amount of volatile compound evolved from finely ground black zira during 30 min of incubation.

strength of cuminaldehyde. The strong antifungal activities of cuminaldehyde and *p*-cymene were further confirmed by testing them against three other phytopathogenic fungi, *B. cinerea*, *V. dahliae*, and *A. mali*.

The inhibition effect of *p*-cymene and cuminaldehyde vapor phase concentration on the mycelial growth of four phytopathogenic fungi was determined (Fig. 3). Cuminaldehyde had significantly stronger mycelial growth inhibition (more than 85%) than *p*-cymene against *F. oxysporum*, *V. dahliae*, and *A. mali* in the 41-mm wells, but this decreased at the 92-mm well. Mycelial growth inhibition of *p*-cymene against all four fungi was more than 40% in each well.

The activities of cuminaldehyde and *p*-cymene against *F. oxysporum* were evaluated based on their $EC_{50,}$ and total activity. The EC_{50} against *F. oxysporum* was 99.7 ppm for *p*-cymene and 0.0312 ppm for cuminaldehyde, respectively. Total activity was 0.0432 for *p*-cymene and 71.5 for cuminaldehyde, which is 1,700 times larger than that of *p*-cymene.

Discussion

The dish pack method, which was originally designed to evaluate the effects of volatile compounds on seed germination, was applied in this study to evaluate antifungal activity.

Table 3 Mycelial growth inhibition against *Fusarium oxysporum* and vapor phase concentrations of authentic compounds detected from black zira

Compound	Vapor phase		Mycelial growth inhibition (%)[b]		
	concentration (ppm v/v)[a]	41 mm[c]	58 mm	82 mm	92 mm
γ-Terpinene	101	18.3±1.5a	9.8±1.6a	7.5±1.4a	2.5±0.8a
p-Cymene	122	56.2±4.8b	51.3±3.8bc	48.0±4.5b	48.0±4.5b
Limonene	183	38.7±5.1c	35.8±4.2bd	26.1±8.0ac	31.8±6.5bc
β-Pinene	896	41.8±3.1bc	37.9±2.6bd	37.3±2.4bc	33.8±2.8bc
α-Pinene	861	46.1±2.9bc	43.0±4.1bd	34.4±4.5bc	33.6±3.5bc
Cuminaldehyde	0.147	92.0±2.5d	66.7±6.3c	23.2±5.7ac	18.1±4.5ac
Myrcene	209	33.3±2.5ac	32.6±2.4d	27.3±2.7ab	26.8±3.4c

[a] Vapor-phase concentration on the 41-mm well after 1 day
[b] Values are means±SE. The average value of the two 41-mm wells were taken and used as one replicate. Values with different letters in each distance are significantly different ($P<0.05$) according to Tukey's test.
[c] Well distance from authentic compound

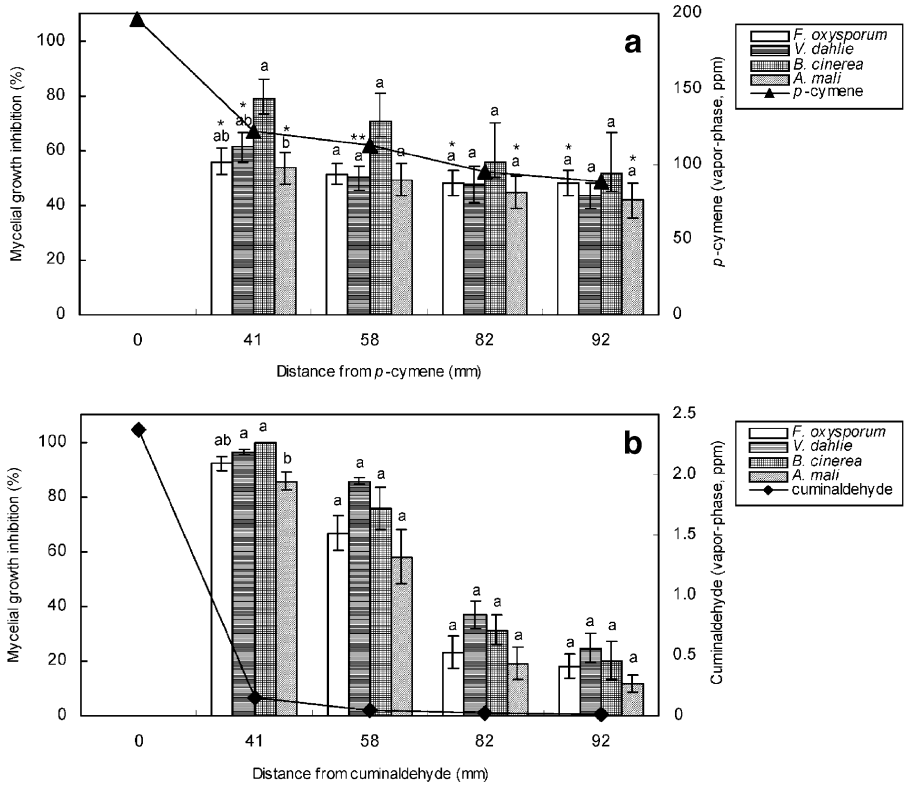


Fig. 3 Effect of *p*-cymene (**a**) and cuminaldehyde (**b**) vapor phase concentrations on the mycelial growth inhibition of four phytopathogenic fungi. Each value represents the mean of four replicates. *Bars* represent standard error of the mean. For each compound, the same letters at each distance indicate that the values are not significantly different ($P<0.05$) according to Tukey's test. *Asterisks* in **a** indicate significant differences between *p*-cymene and cuminaldehyde on the same fungus and in the same well according to Mann–Whitney's *U* test (*asterisk*, $0.01 \leq P < 0.05$, *double asterisk*, $P<0.01$)

The speed of diffusion and intensity of activity of the volatile compounds were estimated based on the relationship of the growth inhibition of the mycelial disk and its distance from the source well. Although black zira showed significant strong mycelial growth inhibition at the 41-mm wells over the two other samples, its inhibition activity sharply decreased as the wells were further distant from the source well. This indicated the low volatility of the volatile(s) in black zira. Beyond the 41-mm-distance wells, the differences in inhibition among the three samples were not significant.

Black zira, which had the strongest activity in the 41-mm wells against *F. oxysporum*, contained seven volatile compounds, γ-terpinene, limonene, *p*-cymene, β-pinene, α-pinene, cuminaldehyde, and myrcene. Baser et al. (1997) analyzed the essential oils of a black zira originating from Tajikistan, and they detected 22 compounds, including *p*-mentha-1,4-dien-7-al, γ-terpinene, β-pinene, and cuminaldehyde, although *p*-mentha-1,4-dien-7-al was not detected in our analysis. This may indicate that our black zira (of Iranian origin) does not contain *p*-mentha-1,4-dien-7-al or that the volatile quantity of this compound was too low to be detected by our analysis procedures.

The mycelial growth inhibition rate of authentic cuminaldehyde declined sharply in the wells farther away from the source well, a characteristic similar to that exhibited by black

zira. On the other hand, the effect of authentic *p*-cymene declined gradually, suggesting that *p*-cymene spread through the multidish faster than cuminaldehyde, resulting in a smaller concentration gradient across the wells.

Evaluation of allelochemicals based on their specific activities (biological activity per unit weight of the compound) expressed as $EC_{50,}$ which is the effective concentration of the compound to induce half-maximum action, is important for the purposes of developing pesticides. If a compound exhibits a small EC_{50} value, it means it has a high specific activity. On the other hand, evaluation by total activity (biological activity per unit weight of the sample containing the bioactive compound) is important for biological uses (Fujii and Hiradate 2005; Hiradate, 2006). By using the concept of total activity, for example, Hiradate et al. (2004) isolated novel plant-growth inhibitory compounds from *Spiraea thunbergii*. In our study, the comparison of the EC_{50} values of both compounds indicated that cuminaldehyde showed higher specific activity, i.e., higher antifungal activity at lower concentration, than *p*-cymene. Furthermore, evaluation by total activity indicated that the activity of cuminaldehyde contained in black zira was more than 1,700 times stronger than *p*-cymene. Therefore, this suggests that the main antifungal compound of black zira is cuminaldehyde.

The strong mycelial growth inhibition by cuminaldehyde against the four phytopathogenic fungi is a good indication of its potential use in disease control. Soylu et al. (2006) also reported that volatiles of essential oils obtained from oregano and thyme inhibited fungal growth of *Phytophthora infestans*, the phytopathogen of tomato late blight disease. The possibility of controlling diseases by using natural volatile compounds from plants is not limited to aromatic species (Wilson et al. 1987; Hamilton-Kemp et al. 1992; Archbold et al. 1997). If the use of natural volatiles with antifungal activities as pest control materials could be established through further screening and research, it could lead to a large reduction in the use of chemical pesticides and labor.

With its high total activity, cuminaldehyde may have a strong influence on the microflora in the neighborhood of black zira in nature. So far, only a few reports regarding plant–microbe interactions through SMs at the community level are available. Karamanoli et al. (2000, 2005) reported the influence of SMs on bacterial colonization on the phyllosphere. Vokou and Liotiri (1999) reported interactions between soil microbes and essential oils. The amount of volatiles from plants generally ranges approximately from 5 to 40 ppt but could reach 50–1,200 ppt in the case of areas surrounded with flowers or in plant communities (Shibuya, 1990). It is likely that under these conditions, volatile compounds influence the growth of nearby microorganisms.

In summary, the antifungal activities of volatile compounds from 52 species of spices and herbs were evaluated by applying the dish pack method. Black zira showed the strongest activity against all four phytopathogenic fungi tested and contained seven volatiles compounds, detected through headspace sampling and GC-MS analysis. Bioassays with authentic compounds showed cuminaldehyde as the main antifungal component of black zira. Natural antifungal volatiles like cuminaldehyde may be used in developing new alternative fungicides that are safe for humans, animals, and the environment. Additionally, our study has demonstrated the effectiveness of the dish pack method in evaluating the antifungal activities of volatiles from spices and herbs, and it could be useful for future investigations of other plant volatiles.

Acknowledgments This work was supported by a Research Fellowship of the Ministry of Agriculture, Forestry, and Fisheries of Japan. We thank Ms. Pariasca for her critical reading of the manuscript.

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Introduction

The heartwood of trees has long been known to produce and accumulate biologically active compounds from a variety of chemical classes (Rennerfelt and Nacht 1955; Rudman 1962, 1963; Scheffer and Cowling 1966) that protect them from microbial decay. Alaska yellow-cedar (*Callitropsis nootkatensis*; previously *Chamaecyparis nootkatensis*; Little et al. 2004; Little 2007) heartwood is an excellent example. Even after these trees die, defensive heartwood chemicals continue to protect standing snags for up to a century in the coastal forests of southeast Alaska and western Canada (Hennon et al. 1990, 2005, 2007a). Changes in snag heartwood chemistry are minimal until after the protective bark sloughs off, and the sapwood is lost from decay and weathering, about 26 years after death (Kelsey et al. 2005). Direct exposure of the snag heartwood to abiotic environmental conditions results in a continuous decline in the concentration of protective chemicals (Kelsey et al. 2005) and eventually a reduction in decay resistance, about 80 years after death (Degroot et al. 2000; Hennon et al. 2007b).

The presence of these strong antimicrobial compounds in yellow-cedar heartwood and its greater resistance than Port-Orford-cedar (*Chamaecyparis lawsoniana*) to the root disease, *Phytophthora lateralis* (McWilliams 2000), were the basis for our previous testing of its heartwood and select components against *P. ramorum* (Manter et al. 2006). *P. ramorum* is an exotic pathogen (Ivors et al. 2006) that causes the stem canker disease known as sudden oak death, which has killed thousands of tanoak (*Lithocarpus densiflorus*), coast live oak (*Quercus agrifolia*), California black oak (*Q. kelloggii*), and Shreve's oak (*Quercus parvula* var. *shrevei*) in coastal counties of California and one county in southwest Oregon since 1995 (Rizzo et al. 2002a, b; Garbelotto and Rizzo 2005; Goheen et al. 2006a). In Europe and the UK, this pathogen is primarily in nurseries or seminatural managed gardens (Werres and De Merlier 2003). It can also cause nonlethal shoot blight or leaf blight in various hosts (Goheen et al. 2006a). The list of proven and suspected hosts has grown to over 50 genera of predominately trees and shrubs (updated list at http://www.aphis.usda.gov/ppq/pramorum). The potential ecological and economic impacts from *P. ramorum* could be extensive depending on its level of direct or indirect influence on nurseries or the composition, structure, diversity, and function of native forests (Rizzo et al. 2005; Goheen et al. 2006a). Management of this pathogen focuses on slowing its spread by preventing introductions and establishment into new areas (Garbelotto and Rizzo 2005; Rizzo et al. 2005). As spores are the key dispersal mechanism (Davidson et al. 2005), control measures that target spore survival may contribute to successful management actions.

Previously, we demonstrated that wood chips, essential oil, and four individual compounds (nootkatin, carvacrol, nootkatone, and valencene) in yellow-cedar heartwood were fungitoxic to *P. ramorum* zoospores and sporangia and fungistatic against hyphae in culture assays (Manter et al. 2006). Microscopic observations detected rupturing of the zoospore outer membrane, and disruption, or release of the cellular contents from developing sporangia before zoospore formation. In addition, heartwood essential oils from incense cedar (*Calocedrus decurrens*), western redcedar (*Thuja plicata*), Port-Orford-cedar (*Chamaecyparis lawsoniana*), and western juniper (*Juniperus occidentalis*) were tested. All four essential oils killed zoospores and inhibited hyphal growth just as strongly as the oil from yellow-cedar, but their heartwood constituents were not further investigated until now. The main objectives of this study were to test the procedure described by Kuhajek et al. (2003) as a potential bioassay technique for antimicrobial activity against *P. ramorum* and to test the antimicrobial activity of individual compounds (most of them commercially available) present in the heartwood of the other conifer

species with antimicrobial activity against *P. ramorum*. In addition, we conducted a field experiment to determine if heartwood chips can limit the proliferation of *P. ramorum in situ*.

Methods and Materials

Heartwood Extracts Tested An initial study was conducted to identify heartwood extracts with antimicrobial activity against *P. ramorum*. We obtained bulk samples of heartwood from single trees of seven conifer species at the locations listed in Table 1. After air drying, each sample was mechanically chipped and then ground in a Wiley mill to pass a screen with 4 or 6 mm openings and finally through a 20 mesh screen. Extracts were prepared in triplicate by weighing 1.0 g of ground heartwood into a glass vial followed by 10.0 ml of ethyl acetate (99.9%, Fisher Scientific). Sealed extracts were kept at room temperature for 24 h with periodic swirling and then filtered through paper (Whatman No. 1 qualitative) into a storage vial.

Compound Identification and Quantification in Heartwood Extracts Volatile constituents in the extracts from incense cedar, western redcedar, Port-Orford-cedar, and western juniper were identified by gas chromatography–mass spectrometry (GC–MS) using a Hewlett Packard 5890 Series II gas chromatograph, with a 5970 mass selective detector and J&W Scientific DB-5 column (30 m×0.25 mm×0.25 μm film thickness). Helium was the carrier gas, with a 1.0 ml min^{-1} flow through the column at 100°C and a 1:10 split. Injector and detector temperatures were 250°C. The oven program started a 100°C for 1.0 min, then increased at 5°C min^{-1} to 150°C, followed by an increase of 3°C min^{-1} to 220°C, with no final hold. Instrument conditions for the analysis of western redcedar extracts were modified to those used by Taylor (2004). The injector and detector were set at 275°C, with an initial column temperature of 100°C and no hold, then increased at 5°C min^{-1} to 130°C, followed by an increase of 30°C min^{-1} to 250°C with a 7.0 min hold. Helium flow through the column was 2.0 ml min^{-1} at 100°C with a 1:10 split. Extracts of western redcedar, western juniper, and Port-Orford-cedar were concentrated by gently evaporating 50–75% of the solvent with a steam of compressed air. Two microliters of these concentrates, or the original incense cedar extract, was injected. Compounds were tentatively identified by comparison of their spectra and relative retention times with those in the Wiley and Adams

Table 1 The antimicrobial activity of ethyl acetate extracts from the heartwood of seven western conifer trees toward *Phytophthora ramorum*

Species	Log_{10} EC_{50} (ppm)[a]	Tree\heartwood Source
Strong inhibitors		
Incense cedar	2.74 (0.08)a	Warm Springs, OR
Western red cedar	2.78 (0.06)a	Alsea, OR
Moderate inhibitors		
Yellow-cedar	3.18 (0.08)b	Prince of Wales Island, AK
Western juniper	3.27 (0.11)b	Warm Springs, OR
Port-Orford-cedar	3.29 (0.09)b	East of Orick, CA
Weak or no activity		
Douglas-fir	>4.00	Warm Springs, OR
Redwood	>4.00	Fortuna, CA

[a] Mean (standard error) from three replicate trials. Means with different letters are significantly different ($P<0.05$).

(2007) libraries and authentic samples of the compounds tested for antimicrobial activity (Table 2). Constituents in yellow-cedar heartwood extracts were recently identified by GC–MS (Kelsey et al. 2005) and were recognized by their relative retention times.

Compound concentrations in the extracts were determined on the same GC with an flame ionization detector (FID) detector and Phenomenex ZB-5 column (30 m×0.25 mm× 0.25 μm film thickness), which is equivalent to the DB-5. The instrument operating conditions were the same as above including the modified settings for western redcedar. Before injection, an internal standard solution containing known quantities of limonene or fenchone in ethyl acetate (Fisher Scientific 99.9%) was added to each extract (1:4, standard/extract measured with analytical syringes). Limonene was the standard for yellow-cedar and fenchone the standard for western juniper, Port-Orford, incense, and western redcedar. Two microliters of each extract/standard mixture was injected. Mean compound concentrations are reported from the three technical replicates of each heartwood extract, except western juniper that had just two replicates.

Individual Compounds Bioassayed Thirteen different volatile compounds were identified from the GC–MS analysis of the conifer heartwood extracts and were selected for further testing. Selected compounds were limited to those that were soluble in ethyl acetate and commercially available or previously isolated in our laboratory (Table 2). Compound purity was determined by transferring 1–2 mg to 1.0 ml of ethyl acetate and analyzing 2μl by GC–FID as described above for compound quantitation. Preliminary studies showed that ethyl

Table 2 The antimicrobial activity, purity as tested, and source for individual compounds tested in *Phytophthora ramorum* bioassays and found in the heartwood extracts of one or more western conifer species

Compound	Log_{10} EC_{50} (ppm)[a]	Purity (%)	Class[b]	Source[c]
Strong inhibitors				
Hinokitiol	0.30 (0.06)a	99.7	T	TCI America
Thymoquinone	0.78 (0.08)b	99.9	M	Acros
Nootkatin	1.00 (0.09)b	99.6	T	Yellow-cedar isolation
Moderate inhibitors				
Nootkatol	1.63 (0.09)c	66.6	S	Lab synthesis
Carvacrol	1.81 (0.08)c	99.4	M	TCI America
Valencene-11,12-diol	1.82 (0.08)c	90.2	S	Yellow-cedar isolation
Weak inhibitors				
α-Terpineol	2.36 (0.07)d	84.1	M	TCI America
Valencene-13-ol	2.38 (0.11)d	55.2	S	Yellow-cedar isolation
(+)-β-Cedrene	2.75 (0.10)e	96.7	S	Fluka (Sigma -Aldrich)
(−)-Thujopsene	2.92 (0.10)e	96.9	S	Fluka (Sigma -Aldrich)
No activity				
(+)-Cedrol	>3.00	99.7	S	Fluka (Sigma -Aldrich)
δ-Cadinene	>3.00	85.5	S	Florida Chemical
Methyl carvacrol	>3.00	98.5	M	Fluka (Sigma -Aldrich)

[a] Mean (standard error) from three replicate trials. Means with different letters are significantly different ($P<0.05$).

[b] Compound class: *T* tropolone; *M* monoterpene; *S* sesquiterpene

[c] For details of isolation and identification of yellow-cedar compounds, see Xioung (2000) and Khasawneh (2003). Nootkatol synthesized from sodium borohydride reduction of nootkatone

acetate exhibited no antimicrobial activity against *P. ramorum* at concentrations less than or equal to 1% (data not shown).

Bioassays Antimicrobial activity of heartwood extracts or individual compounds was measured with the photometric assay developed by Kuhajek et al. (2003) for bioassays with *Phytophthora* spp., but not tested with *P. ramorum*. Zoospores from one isolate of *P. ramorum* (A1 mating type, obtained from an infected rhododendron plant at a commercial nursery in Clackamas, Oregon) were obtained by flooding 2-week-old cultures grown on clarified V8 agar plates (66.7 ml of V8 juice and 2 g $CaCO_3$, filtered through cheesecloth, plus 15 g of agar [Bacto-agar, Difco Laboratories, Detroit, MI, USA] per liter of distilled water) with 10 ml H_2O and incubating at 4°C for 4 h. Zoospore solutions were quantified by direct counting with a hemocytometer (×40 magnification) and diluted to appropriate concentrations with dH_2O. One isolate was considered adequate as six *P. ramorum* isolates (A1 and A2 mating types) responded similarly in previous bioassays (Manter et al. 2006). Growth of *P. ramorum* was evaluated in a 96-well microtiter format by measuring optical density at 650 nm (OD_{650}) at 0, 16, 24, 48, and 72 h with a microplate reader (Vmax Kinetic Microplate Reader, Molecular Devices, Menlo Park, CA, USA). Between measurements, plates were covered and incubated at room temperature with gentle shaking at 80 rpm. All OD_{650} values were corrected for background absorbance at time zero. The effect of starting zoospore concentrations on growth curves was analyzed by adding 50µl of a zoospore solution (0, 10, 100, 1,000, 2,500, 5,000, 7,500, or 10,000 zoospores ml^{-1}) and 100µl of clarified V8 broth to each of six replicate wells. Antimicrobial activity of all test compounds was analyzed by adding 50µl of a zoospore solution (10^4 zoospores ml^{-1}) and 100µl of V8 broth amended with an individual test compound to each of six replicate wells. All heartwood extracts were tested at 0, 10, 50, 100, 500, 1,000, 5,000, and 10,000 ppm (*v*/*v*), and all individual compounds were tested at 0, 1, 5, 10, 50, 100, 500, and 1,000 ppm (*v*/*v*). Additional concentrations at 25 ppm were included for hinokitiol, nootkatin, nootkatol, carvacrol, and valencene-11,12-diol and at 250 ppm for α-terpineol and valencene-13-ol. Each experimental plate also included six replicate sterility control wells (100µl of V8 broth and 50µl dH_2O). Plates with growth in the sterility control wells were discarded from the analysis. Each compound and concentration combination was replicated ×3, and a Log_{10} EC_{50} was determined for each trial as described below.

Heartwood Field Trial To determine whether chips of redcedar heartwood can limit *P. ramorum* buildup in soil and litter under natural conditions, we conducted a field test on the Rush Creek Open Space Preserve, at Novato, California (38°7.22′N, 122°33.70′W), in the spring and early summer of 2006. A fresh bulk sample of redcedar heartwood was collected in January 2006 from the same tree sampled for the laboratory bioassays reported in Table 1. The wood was cut into pieces, air dried in the laboratory, and chipped (1–3 cm long, 0.5–3 cm wide, 0.1–0.5 cm thick). Redwood was selected as a control because it was inactive in the laboratory bioassays. Kiln-dried and planed redcedar boards were obtained from a commercial source in Eugene, Oregon in January 2006 and chipped in our laboratory. Water content was measured on six subsamples of each species by oven drying them for 16 h at 102°C and cooling to room temperature in a desiccator box before reweighing. The dry weight equivalent of 140 g of fresh chips was enclosed in a nylon mesh bag (approx. 20×20 cm, 2.5×3.5 mm hole) sealed on the edges with cloth tape and staples.

On March 1, 2006, ten *Umbellularia californica*, California bay laurel, trees with leaves symptomatic of *P. ramorum* infection (Goheen et al. 2006a) were selected to serve as

blocks on the northwest side of Pinheiro ridge in the Rush Creek Preserve. Two plots (1× 1 m) were located beneath each tree where the canopy had the most symptomatic leaves. Six bags of redcedar chips were randomly positioned on one plot (selected from a 5×5 grid of 20×20 cm subplots) and six bags of redwood on the other plot. The bags were placed on top of the litter and secured with long wire pins on each corner penetrating into the soil to minimize movement by passing animals. On two sample dates (2 months posttreatment: May 2, 2006; 4 months posttreatment: June 29, 2006), three randomly chosen subplots were sampled from each plot. Bags were lifted carefully, and the litter beneath the center of the bag was gathered. We wore plastic gloves that were changed after each sample to minimize cross contamination. The litter depth varied tremendously; in some instances, it was only one or two leaves deep, and on other sites, it was 1–2 cm or greater. Although leaves of *U. californica* were usually present, leaves from other tree species nearby or grass blades were also common and were included in the sample that was sealed in a glass scintillation vial. Bits and pieces of surface soil may have adhered to leaves in direct contact with the soil. A plug of soil beneath the litter was taken to a depth of 3.0 cm with a #8 cork borer (1.4-cm diameter) and sealed in a glass scintillation vial. The borer was cleaned between samples with a 70% isopropyl alcohol swab and dried with a fresh tissue to reduce cross contamination.

Analysis of Phytophthora ramorum Proliferation by Quantitative Polymerase Chain Reaction Soil and litter samples from the Rush Creek Preserve were shipped back to the laboratory on ice and stored at −20°C until DNA extraction. Total genomic DNA was extracted from each sample by using Ultraclean Soil or Plant DNA kits (MoBio, Carlsbad, CA) per the manufacturer's protocol. DNA from the litter samples was further purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Amplification of *P. ramorum* DNA was performed by using the species-specific nested protocol developed by Hayden et al. (2006). The amount of *P. ramorum* DNA present in each sample was calculated from measured cycle threshold values (BioRad, iCycle, Hercules, CA, USA) and an external standard curve generated from serially diluted *P. ramorum* genomic DNA isolated from pure cultures. All reported values of *P. ramorum* DNA are per unit dry weight of soil or litter extracted. The three subsamples (bags) from each plot were averaged, and treatment differences were analyzed by analysis of variance (ANOVA) as described below.

Data Analysis Kuhajek et al. (2003) suggested that bioactivity can be determined from the ratio of treatment and control OD values at a particular time point. However, unless a linear relationship between OD and initial zoospore concentration exists at the chosen time point, this technique will lead to an inaccurate estimate of activity. In our study, we did not observe a linear relationship at any of the selected time points (data not shown); furthermore, activity estimates will vary depending upon the chosen time point, if the slope of the OD vs. initial zoospore concentration regression line changes. To minimize these problems, the following analysis procedures were used. For each concentration of extract or compound bioassayed, a sigmoidal curve was fitted to the corrected OD_{650} growth curves (e.g., Fig. 1a) to obtain an estimate of $time_{1/2}$ or time (h) it takes to reach the midpoint between minimum and maximum OD_{650} values. This calculated parameter ($time_{1/2}$) was linearly related to initial zoospore concentration (Fig. 1b) and has the advantage that it is independent of the specific time points included in the analysis. Antimicrobial activity of each compound was then determined by fitting a sigmoidal curve to the $time_{1/2}$ vs. Log_{10} concentration (ppm) of each test compound and calculating the Log_{10} EC_{50} from this curve.

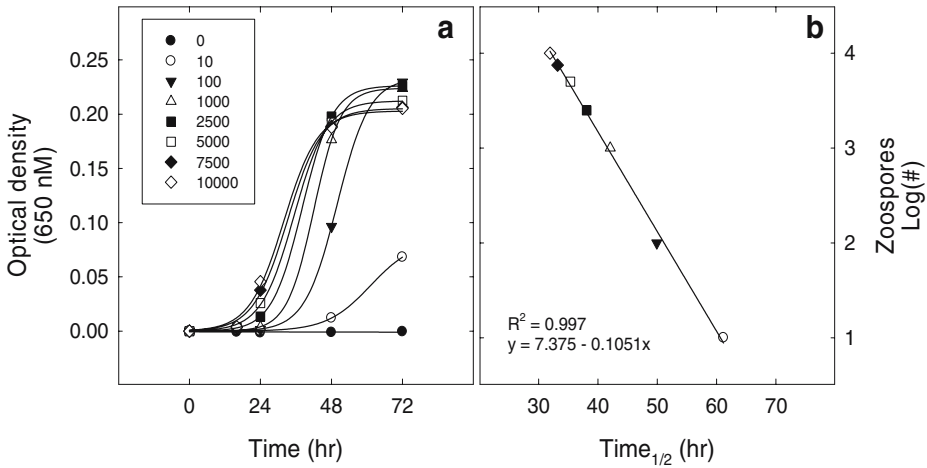


Fig. 1 Changes in optical density (OD_{650}) over time for microplate well cultures of *P. ramorum* initiated with different zoospore concentrations (number ml^{-1}; **a**) and the relationship between starting zoospore concentrations on a log_{10} scale and the well culture OD_{650} midpoints, or $time_{1/2}$ (**b**)

All regressions were performed by using SigmaPlot 9.0 (Systat Software Inc., Point Richmond, CA, USA). Significant differences between treatments (compounds) were tested by ANOVA and Fisher's least significant difference post hoc comparison ($P<0.5$) using SAS Vers 9.1 (Cary, NC).

P. ramorum DNA concentrations in the litter beneath the redcedar and redwood chips after 0, 2, or 4 months of treatment were analyzed as a randomized complete block design with ten replications by ANOVA using SAS Ver. 9.1. Data were natural log transformed to meet the assumption of homogeneous variance, and back-transformed least squares means and pooled standard errors are presented. Differences among means were determined by Fisher's protected least significant difference ($P<0.05$). The DNA concentrations in soil samples were analyzed separately using the same analysis procedure.

Results

Heartwood extracts from the trees sampled were grouped into three levels of antimicrobial activity: (1) strong—incense cedar and western redcedar; (2) moderate—yellow-cedar, western juniper, and Port-Orford-cedar, and (3) weak or no activity—Douglas-fir (*Pseudotsuga menziesii*) and redwood (*Sequoia sempervirens*) (Table 1). Individual compounds were separated into four activity categories: (1) strong (Log_{10} $EC_{50}<1.0$ ppm)—hinokitiol, thymoquinone, nootkatin; (2) moderate—(Log_{10} EC_{50} 1.0–2.0 ppm) nootkatol, carvacrol, valencene-11,12-diol; (3) weak (Log_{10} EC_{50} 2.0–3.0 ppm)—α-terpineol, valencene-13-ol, β-cedrene, and thujopsene; and (4) no activity (Log_{10} $EC_{50}>3.0$ ppm)—cedrol, δ-cadinene, methylcarvacrol (Table 2).

The volatile compounds identified in each extract are shown in Table 3 and were distinct for each tree (species), except for a few compounds. Carvacrol was detected in three trees—incense cedar, western redcedar, and yellow-cedar. α-Terpineol and δ-cadinene were both present in western redcedar and Port-Orford-cedar. Constituents in the Douglas-fir and

Table 3 Compound concentrations in heartwood extracts tested for antimicrobial activity toward *Phytophthora ramorum*

No.	Compound[a]	Incense Cedar[b]	Western Redcedar	Yellow-cedar	Western Juniper	Port-Orford-cedar
1	*Carvacrol*	189.5	4.4	77.4		
2	Heyderiol	673.0				
3	Libocedrol	186.1				
4	*p*-Methoxythymol	479.6				
5	*p*-Methoxycarvacrol	129.9				
6	*Thymoquinone*	193.3				
7	Thymohydroquinone	66.4				
8	*Hinokitiol*		12.8			
9	Myrtenol		7.0			
10	Nezukone		32.0			
11	Terpinen-4-ol		5.0			
12	α-*Terpineol*		5.9			374.1
13	Thujic acid		76.6			
14	*δ-Cadinene*			10.7		740.4
15	*Methyl carvacrol*			4.9		
16	Nookatone			74.4		
17	Nootkatene			126.0		
18	*Nootkatin*			55.0		
19	*epi*-Nootkatol			17.1		
20	*Nootkatol*			29.3		
21	Valencene			18.9		
22	*Valencene-13-ol*			28.4		
23	*Valencene-11,12-diol*			44.6		
24	α-Cedrene				62.6	
25	*Cedrol*				288.0	
26	*β-Cedrene*				14.9	
27	*Thujopsene*				15.3	
28	α-Amorphene					151.3
29	Borneol					93.9
30	α-Cadinene					57.7
31	γ-Cadinene					395.2
32	α-Cadinol					934.4
33	Fenchol					57.0
34	α-Muurolene					288.8
35	*epi*-α-Muurolol					860.6
36	α-Terpenyl acetate					65.2
37	α-Muurolol					168.4
38	Unknowns	82.1 (2)[c]	48.4 (7)		412.4 (7)	177.0 (1)
	Total	1,999.9	192.1	486.7	793.2	4,364.0

[a] Compounds (μg ml^{-1}) listed alphabetically within species, except those in multiple species. All compounds were identified by comparison of their spectra and retention times with those in the Wiley and Adams (2007) libraries. Those in *italicized font* were also compared with the authentic samples tested individually for antimicrobial activity (Table 2).

[b] Identities of *p*-methoxythymol and *p*-methoxycarvacrol were assigned based on molecular weights (Zavarin and Anderson 1955) and molecular ions in their mass spectra and relative retention times for thymol and carvacrol provided by Adams (2007).

[c] Number of unknown peaks in parentheses.

redwood extracts were not analyzed because they exhibited no antimicrobial activity. Two or more of the individual compounds tested for antimicrobial activity were present in the extracts that exhibited bioactivity (Table 3).

P. ramorum DNA was present at detectable levels in all soil and litter samples on both sample dates. For both heartwood chip treatments, *P. ramorum* DNA increased over time with the greatest increases in the litter layer. On May 1, 2 months after placing the chips on the forest floor, there was no difference between treatments in the amount of *P. ramorum* DNA in the litter (Fig. 2a). Between May 1 and June 29, *P. ramorum* DNA on the redwood control plots increased 11.1 times, compared with only 2.6 times on the redcedar plots, resulting in the redwood plots having 4.3 times more *P. ramorum* DNA than the redcedar (Fig. 2a). Soils from redwood and redcedar plots had the same quantities of DNA in May and June (Fig. 2b).

Discussion

The new protocol tested here and developed initially by Kuhajek et al. (2003) is easier to use, allows more samples to be quickly analyzed, and requires much smaller amounts of media and test materials than the bioassay procedure used previously for testing the activity of yellow-cedar compounds toward *P. ramorum* (Manter et al. 2006). Disadvantages include the inability to directly test heartwood activity because of interference with light transmittance. Furthermore, it does not distinguish inhibition or disruption of zoospore or sporangia germination from inhibition of hyphal growth. Our previous results suggest that the key mode of action for many of the toxic yellow-cedar compounds is lysis of zoospore and sporangia cell membranes (Manter et al. 2006). The antibacterial activity of essential oils and individual oil constituents, including carvacrol, has been attributed to their hydrophobicity, which allows them to enter the cell or mitochondrial membranes (reviewed by Burt 2004). This disruption increases membrane permeability and leakage of the cellular contents. Hinokitiol, a redcedar component, also causes cell lysis for some Gram-negative bacteria (Trust and Coombs 1973) and can rupture cercariae of *Schistosoma mansoni* (Chisty et al. 2004). However, regardless of the mechanism for reducing growth (i.e.,

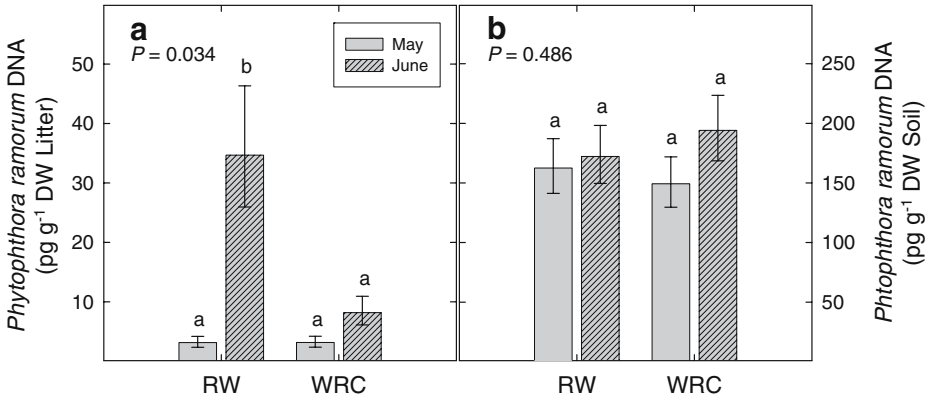


Fig. 2 Concentrations of *P. ramorum* DNA in the litter (**a**) and soil (**b**) after 2 and 4 months treatment with western redcedar (*WRC*) or redwood (*RW*) chips beneath the canopy of *U. californica* trees with leaves showing symptoms of *P. ramorum* leaf blight. *Bars* represent the median and pooled standard errors resulting from back-transformations. *Bars with different letters* are statistically significant at $P<0.05$

disruption of zoospore or sporangia membranes and/or limiting hyphal growth rates), compounds with antimicrobial activity will be detected by the photometric methodology used in this study. Nootkatin and carvacrol were tested here and with our earlier procedure (Manter et al. 2006). In both instances, nootkatin was more active than carvacrol. Previously, nootkatin had a Log_{10} EC_{50} of 0.18 and 0.84 ppm for inhibition of zoospore and sporangia germination, respectively, compared with 1.03 and 1.85 ppm for carvacrol (Manter et al. 2006). In the current assay, the Log_{10} EC_{50} for nootkatin was 1.00± 0.09 ppm, and carvacrol was 1.81±0.08 ppm.

Of the seven different conifer heartwood extracts bioassayed in the initial experiment, those from western redcedar and incense cedar were more than twice as active (less than half of the EC_{50}) toward *P. ramorum* than extracts from the group including yellow-cedar, western juniper, and Port-Orford-cedar (Table 2). Douglas-fir and redwood extracts were inactive. Previously, the steam distilled essential oils from yellow-cedar, western juniper, Port-Orford-cedar, and incense cedar were shown to have nearly identical levels of activity toward *P. ramorum* zoospore germination and hyphal growth when tested at 140 ppm (Manter et al. 2006). As the chemical composition of conifer heartwood can be extremely variable among trees (DeBell et al. 1999; Kelsey et al. 2005), our ranking of species activities in Table 2 applies only to the trees used in this study. Confirmation of these species differences will require testing of a larger random sample.

All individually tested compounds with strong or moderate antimicrobial activity contained a free hydroxy group, except for thymoquinone. The importance of this functionality is demonstrated by the tremendous difference in activity between carvacrol (Log_{10} EC_{50} 1.81±0.08 ppm) and methyl carvacrol (>3.0 ppm) with a methyl group blocking the hydroxyl. The antibacterial action of carvacrol against the food pathogen, *Bacillus cereus*, is also dependant on the free hydroxyl (Ultee et al. 2002). In contrast, the cytotoxicity of thymoquinone decreases 1.7-fold when the two ketones are reduced to alcohols (Johnson et al. 1998). Hydroxylated compounds with the lowest antimicrobial activity in our bioassays included two sesquiterpenes (cedrol and valencene-13-ol) and one monoterpene (α-terpineol). Valencene-13-ol and nootkatol expressed weak activity as tested at 55 and 66.6% purity, respectively. Had they been at 95% purity, the valencene-13-ol would probably have increased to the moderate level of activity, and nootkatol might have reach the strong category. Of the three sesquiterpene hydrocarbons tested, β-cedrene and thujopsene were weakly active, and δ-cadinene had no activity toward *P. ramorum*.

All compounds in the strong and moderate categories here have been shown to be active against a range of other organisms. Hinokitiol is probably the most rigorously investigated with activity against human tumor cells and a variety of microorganisms that cause plant disease or decay, and diseases of humans (Trust and Coombs 1973; Fallik and Grinberg 1992; Inamori et al. 1993, 1999; Baya et al. 2001; Arima et al. 2003; Voda et al. 2003; Chisty et al. 2004; Yamano et al. 2005). Studies of carvacrol have demonstrated bactericidal, fungicidal, insecticidal, nematicidal, and acaricidal activities (Rennerfelt and Nacht 1955; Ahn et al. 1998; Oka et al. 2000; Voda et al. 2003; Burt 2004). Nootkatin has antimicrobial properties against wood decay fungi (Rennerfelt and Nacht 1955) and *Candida albicans*, a yeast that causes infections in animals (Johnston et al. 2001). Thymoquinone is cytotoxic for certain human tumor cells and is insecticidal toward yellow fever mosquito larvae (Johnson et al. 1998).

Conifer heartwood typically contains a complex mixture of compounds from various chemical classes and in varying concentrations (Renerfelt and Nacht 1955; Rudman 1962, 1963; Scheffer and Cowling 1966). This study focused on volatile compounds soluble in ethyl acetate and detectable by GC. Not all of the active compounds in these extracts would

be detected by this method, and there could be other active compounds remaining in the wood because of their low solubility in this solvent. This is especially relevant for redcedar because its heartwood contains many nonvolatile phenolics, such as plicatic acid, plicatin, or thujaplicatin (Barton and MacDonald 1971) with weak antimicrobial properties (Roff and Atkinson 1954). Some proportion of these compounds would be removed with ethyl acetate, as they are soluble in acetone (Barton and MacDonald 1971), but they are not detectable by GC. Thus, woody tissues of the conifers tested here could have stronger *in vivo* bioactivities than their extracts demonstrated *in vitro*. Despite our limited search of the chosen conifer metabolomes, we successfully identified a number of highly active, commercially available compounds that provide strong activity against *P. ramorum* in culture.

Results from the field trial with chips of western redcedar and redwood were consistent with *in vitro* bioassays of their heartwood extracts. After just 2 months of treatment, there was no difference in the *P. ramorum* DNA or biomass in the litter beneath the redwood or western redcedar chips. In the subsequent 2 months, however, *P. ramorum* DNA on the redwood plots increased 4.3 times the amount on the redcedar plots. A combination of rainfall, spore production, and bioactivity of the wood chips may explain these results. Warm winter or spring rains stimulate *P. ramorum* to grow and sporulate on wet leaf surfaces of suitable hosts, especially bay laurel. Once zoospores or chlamydospores are produced on the leaves, subsequent rainfall washes them to new leaves or the forest floor. Upon installation of the field plots at the beginning of March, there were relatively few leaves on the bay laurel with visible symptoms. During March and April, the site experienced heavy rainfall, 205 and 127 mm, respectively (California Climate Data Archive [http://www.calclim.dri.edu/ccda/ncacoop.html], weather station 046826 located 16 km North of the field site). At the first sampling date in early May, the number of visibly infected bay laurel leaves had increased substantially in the lower canopy. In May and June, rainfall decreased to 8 and 0 mm, but the level of bay laurel canopy infection had expanded further by the last collection date on 29 June.

In the absence of woodchips covering the forest floor, Davidson et al. (2005) observed a 2- to 4-month lag between peak rainfall and detection of *P. ramorum* in the litter and soil. The low levels of *P. ramorum* DNA in the two chip treatments of the May sample are thus not unexpected because of the time lag between rainfall and spore deposition. Additionally, the presence of a layer of woodchips, with or without antimicrobial activity, could physically trap and reduce spore movement to the litter layer below. Furthermore, spores directly contacting the western redcedar wood, or its leachate, would be exposed to antimicrobial compounds that can damage spore membranes and stop their further movement to the litter layer. It is also possible that chlamydospores present in the litter before treatment germinated and formed new sporangia and zoospores in response to the heavy March and April rainfall. Here again, those exposed to the antimicrobial compounds of western red cedar would experience lower germination and growth rates compared to those in the redwood treatment, which had no antimicrobial activity. An alternative interpretation of the field data is that western redcedar had no impact on *P. ramorum*, and the redwood treatment stimulated its growth in the litter. This seem unlikely given the strong activity of western redcedar extracts *in vitro* and the observation that direct inoculations of logs and stems of both redwood and western redcedar showed no differences in the growth of *P. ramorum* (Hansen et al. 2005).

We chose to quantify *P. ramorum* biomass in the field trial by quantitative polymerase chain reaction because of its high reproducibility and sensitivity to detect changes in *P. ramorum* biomass (Hayden et al. 2006; Schena et al. 2006). However, this technique cannot

distinguish between viable and nonviable biomass present in a sample. Although other techniques, such as leaf baiting or direct plating, will detect only viable *P. ramorum* biomass, they are at best semi-quantitative or not species specific. Furthermore, even if some nonviable *P. ramorum* was detected in our samples, it is clear that viable *P. ramorum* is present and is increasing significantly over time under the redwood, but not the redcedar chips. Finally, we suggest that the presence of any nonviable *P. ramorum* in the redcedar plots may be masking our ability to detect the full bioactivity of redcedar chips *in situ*—not only does it limit the proliferation and development of new biomass, but it also kills any *P. ramorum* already present in the system.

Management of this pathogen focuses on slowing its spread by preventing introductions and establishment into new areas (Garbelotto and Rizzo 2005; Rizzo et al. 2005). With spores being the key dispersal mechanism (Davidson et al. 2005), control measures that target spore survival may contribute to successful management actions. An integrated management program may be able to utilize bioactive conifer heartwoods in some instances to help limit the number of *P. ramorum* spores in soils and the potential for new infections via splash dispersal (Davidson et al. 2005) or long-distance transport to new areas. For example, people living, working, and recreating in areas infested with *P. ramorum* can vector spores on their shoes, bicycle tires, or other items that contact spore contaminated soils or plant tissues (Cushman and Meentemeyer 2005; Davidson et al. 2005). Previously, we proposed that chips of yellow-cedar might be spread over trails, bike paths, or parking lots used by recreationists to reduce spore movement and redistribution from these areas (Manter et al. 2006). However, this will not affect movement of viable hyphal fragments.

Alternatively, some of the most active individual heartwood compounds might be developed into useful products. There are several fungicides that can protect against *P. ramorum* infection when applied in advance of inoculation (Heungens et al. 2006; Linderman and Davis 2006; Tjosvold et al. 2006; Turner et al. 2006), but their protection against foliar infection varies with host species, age of leaves, and source of the fungal isolate (Goheen et al. 2006b). Post inoculation applications tend to be less effective, and none of the fungicides is capable of eradicating the pathogen from infected plants (Heungens et al. 2006; Linderman and Davies 2006; Tjosvold et al. 2006). In addition, there are concerns for rapid development of fungicide resistance (Turner et al. 2006). Thus, it may be worthwhile to test additional hydroxylated compounds from conifer species that have not yet been bioassayed and to examine the efficacy of foliar spray applications of the most active compounds such as hinokitiol and thymoquinone.

Acknowledgements We thank the US Department of Agriculture, Forest Service, Pacific Southwest Research Station for funding and the Marin County Open Space District for a permit to work on the Rush Creek Preserve. We thank John Bannister, Ketchikan Wood Technology Center for samples of Alaska yellow-cedar, Gene Braber, USDA, Orleans Ranger District for the sample of Port-Orford-cedar, and Greg Jennings, Bureau of Land Management, Arcata, California Field Office for the sample of redwood. We are especially thankful to Dr. Everett Hansen at Oregon State University for providing the *P. ramorum* isolate and use of his laboratory facilities and to Lyndsay Frady for her assistance conducting the bioassays.

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spot to overwinter. Aggregation pheromones, as well as sex pheromones, are essential components of integrated pest management methods that are used in push–pull strategies to repel the target pests from a protected host, while luring them toward an attractive trap (Cook et al. 2007). Although the multicolored Asian lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), was introduced in Europe and North America as an effective natural enemy of various pests that include aphids and mites, adverse effects of *H. axyridis* on nontarget insects, humans, and crops have been identified. Furthermore, *H. axyridis* negatively impacts native coccinellids, as *H. axyridis* is the top predator in the aphidophagous guild. It occupies the ecological niches of endemic lady beetles and other aphid-specific predators such as the two-spotted lady beetle, *Adalia bipunctata* L., or the seven-spotted lady beetle, *Coccinella septempunctata* L. (Koch 2003; Sato et al. 2005). *Harmonia axyridis* is also considered a nuisance pest because it migrates from the field into houses and other structures when temperatures decline, and forms mass overwintering aggregations in elevated, dark, and concealed portions of structures (Huelsman et al. 2002). In addition to its invasive behavior, *H. axyridis*, when disturbed, secretes hemolymph that imparts an unpleasant odor to any surfaces (walls, furnishings, etc.) where beetles aggregate (Huelsman et al. 2002; Magnan et al. 2002). The secretions and the unpleasant odor can lead to allergic reactions in human occupants.

To develop efficient push–pull strategies, better knowledge is required of the potential semiochemicals involved in the establishment and persistence of overwintering aggregations of *H. axyridis*. While observing *H. axyridis* aggregation sites, Nalepa et al. (2000) concluded that there was little evidence for volatile aggregation pheromones and that contact chemoreception with conspecifics probably mediates the aggregation behavior. However, Brown et al. (2006) reported the identification of (−)-β-caryophyllene from the headspace of *H. axyridis* females under conditions that simulated natural overwintering conditions. (−)-β-Caryophyllene is also commonly released from various plant families, including the Brassicaceae (Rohloff and Bones 2005), Solanaceae (Farag and Paré 2002), and Poaceae (Dean and De Moraes 2006).

To demonstrate the potential role of (−)-β-caryophyllene in the aggregation behavior of *H. axyridis*, and the impact of (*E*)-β-farnesene (major component of the aphid alarm pheromone) on the foraging behavior of this aphid predator, we conducted both electrophysiological and behavioral experiments. These two methodological approaches have been used widely in the identification of lady beetle-related semiochemicals (Zhu et al. 1999; Al Abassi et al. 2000; Acar et al. 2001; Francis et al. 2001, 2004; Ninkovic et al. 2001).

Methods and Materials

Insects Larvae of *H. axyridis* were collected in May 2007 on the edges of a bean field and placed in aerated plastic boxes (up to 25 individuals per container). The larvae and the resulting adults were provisioned daily *ad libitum* with aphids, *Acyrthosiphon pisum* Harris, reared on beans. Sugar, water-impregnated cotton, and multiflower pollen were also provided. Boxes were placed in controlled environment incubators (16-hr-light photoperiod; 25±2°C; 70% RH). Males and females were separated at least 1 wk prior to electrophysiological and behavioral bioassays.

Electrophysiology (EAG) The *H. axyridis* antenna was carefully excised from the head so that all segments and the basal nerve were still attached. The antenna was mounted between two glass Ag–AgCl electrodes (Harvard Apparatus, Holliston, MA, USA; 1.5 mm o.d.×

1.17 mm i.d.) filled with saline solution (NaCl, 7.5 g/l; $CaCl_2$, 0.21 g/l; KCl, 0.35 g/l; $NaHCO_3$, 0.2 g/l) and in contact with a silver wire. The base of the antenna was first inserted into the ground glass electrode. The tip of the recording electrode was bowl-shaped, and half of the last distal antennal segment was immersed into the saline solution. This setup was previously shown to produce elegant results in the study of the olfactory responses of the confused flour beetle, *Tribolium confusum* Du Val (Coleoptera: Tenebrionidae), to its aggregation pheromone (Verheggen et al. 2007). The DC potential was recorded on a computer (Auto Spike v. 3.0) by using an amplifier (IDAC-4, Syntech®, Hilversum, The Netherlands) with 100-fold amplification. A 0.5-cm^2 piece of filter paper that was impregnated with 10 μl of the chemical under examination was placed in a Pasteur pipette and used to puff an air sample in a constant 1.5 l/min airstream. As a negative control, the antennae were first stimulated with semiochemical-free filter paper (=mechanical stimulus). Later, the antennae were stimulated with (−)-β-caryophyllene or (*E*)-β-farnesene (EβF). (−)-β-Caryophyllene was purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany) and had a chemical purity of >97% (determined by GC). EβF was synthesized from farnesol (Tanaka et al. 1975) and had a chemical purity of 98% (also determined by GC). Ten antennae were tested for each sex.

Four-arm Olfactometer Assays The four-arm olfactometer was similar to that previously described by Vet et al. (1983). It was constructed entirely of Teflon® and was closed with a removable glass roof, both previously cleaned with *n*-hexane. The walking arena was 40 cm wide (from center to odor source) and 1.5 cm high (from Teflon® walking arena to glass ceiling). Charcoal-filtered air was pushed in each of the four olfactometer arms through Teflon® tubing and adjusted to 100 ml/min with a digital flowmeter. A pump ventilated the walking arena by removing air from the center at 400 ml/min. A 0.5-l glass chamber was connected to one of the four olfactometer arms and was used to dispose of the odor source. Three stimuli were tested on both *H. axyridis* males and females: (1) the four arms of the olfactometer were connected to pure air sources, the glass chamber remained empty; (2) 20 unwinged adult aphids, *Myzus persicae* Sultzer, were rapidly crushed inside of the glass chamber by using a small glass pestle left inside the chamber [as a natural source of EβF: according to previous studies, the volatiles released by crushed *M. persicae* consist exclusively of EβF (Edwards et al. 1973; Francis et al. 2005)]; (3) a 0.5-cm^2 piece of filter paper was impregnated with 10 μl of (−)-β-caryophyllene and placed inside the glass chamber. The glass chamber was randomly connected to one of the four arms of the olfactometer. Both the walking arena and the glass ceiling were washed with *n*-hexane after each lady beetle was tested. The olfactometer was divided into one central 10-cm squared area and four other areas related to the four odor sources. The choice of the lady beetle was determined by (a) the first area it entered, (b) the first area where it stood for 30 consecutive seconds, and (c) the area where it stood at the end of the 3 min of observation (=last entered area). The behavioral observations were conducted in a laboratory at 22±1°C and under uniform lighting to avoid interference with behavior of the test insects.

Aggregation Assays Three 5-cm-diameter holes were cut into a 30×15×15-cm plastic box, and the holes were covered with metal screening. One hole was located on each lateral side (30 cm apart) and a third hole was located on the top. A 4-cm^2 piece of filter paper impregnated with 10 μl of (−)-β-caryophyllene was attached to the screen cover of one of the lateral holes by using a rubber band. Ten males and 10 females *H. axyridis* were introduced into the box for 1 hr, and the side where they stood was recorded after 30 and 60 min. Behavioral observations were conducted under the same conditions as mentioned above.

Statistical Analyses A Student's *t* test was performed to compare the mean EAG responses to the semiochemical stimuli with the EAG responses to the mechanical stimulant, as well as to compare EAG responses between the sexes. Observed frequencies related to the choice of *H. axyridis* in olfactometer assays (four-arm and aggregation bioassays) were compared to corresponding theoretical frequencies by using a χ^2 goodness-of-fit test. All statistical tests were conducted using Minitab® release 14.2.

Results

Electroantennography To the best of our knowledge, this is the first report of successful EAG recordings from *H. axyridis* antennae. Both pure EβF and (−)-β-caryophyllene elicited good activity from antennae of both sexes. Female and male antennal responses to the latter semiochemical were higher than those produced mechanically (Student's *t* test, t_{obs}=4.89, P<0.001, and t_{obs}=5.49, P<0.001, for females and males, respectively). Male antennae were more sensitive to (−)-β-caryophyllene (338±31 μV, N=10) than female antennae (178±19 μV, N=10) (t_{obs}=4.41, P=0.001).

The mean EAG responses to EβF were significantly higher than those to mechanical stimuli (t_{obs}=2.43, P=0.027, and t_{obs}=2.53, P=0.022, for females and males, respectively). The mean EAG responses to EβF was higher in males (202±17 μV) than in females (120± 18 μV) (t_{obs}=3.30, P=0.004). (−)-β-Caryophyllene induced higher EAG responses than EβF (t_{obs}=2.22, P=0.040, and t_{obs}=3.84, P=0.002, for females and males, respectively).

Four-arm Olfactometer Assays In the four-arm olfactometer, EβF (crushed *M. persicae* as a natural source) and (−)-β-caryophyllene elicited significant behavioral activity from both *H. axyridis* males and females (Table 1). According to the three behavioral criteria that we observed and recorded, males were attracted to (−)-β-caryophyllene. Females were less attracted than males to (−)-β-caryophyllene, as significant results were obtained only while observing the last area entered (χ^2=7.92, P=0.048). EβF attracted both male and female *H. axyridis* (Table 1).

Aggregation Assays After 30 min, both sexes of *H. axyridis* aggregated on the (−)-β-caryophyllene side of the box (χ^2=20.48, P<0.001, and χ^2=18.00, P<0.001, for males and females, respectively) (Fig. 1). Similar results were observed after 1 hr, as both males and females were still aggregated in the side of the box where (−)-β-caryophyllene was released (χ^2=11.52, P=0.001, and χ^2=13.52, P<0.001, respectively).

Discussion

Comprehension of the chemical ecology of *H. axyridis* is essential before developing push–pull strategies for the efficient use of this lady beetle in aphid control or for the control of this new urban pest in structures. (−)-β-Caryophyllene and EβF are ubiquitous plant volatiles, and previous studies have demonstrated the role of plant semiochemicals in the searching behavior of aphid natural enemies (Tumlinson et al. 1992). In our experiments, (−)-β-caryophyllene and EβF elicited antennal activity in both sexes and significant attraction in a four-arm olfactometer. It has been suggested that lady beetles orient

Table 1 Behavioral responses [observed frequencies (N=50) were compared to expected frequencies assuming random distribution (one odor source and three controls) by using a χ^2 test] of *H. axyridis* to two sesquiterpenes

Odor Source	*H. axyridis* Gender	Behavioral Observations	Observed Frequencies	χ^2	P
(−)-β-Caryophyllene	Female	First entered area	0.38	4.56	0.207
		First area entered for 30 sec	0.38	4.56	0.207
		Last entered area	0.42	7.92	0.048
	Male	First entered area	0.44	9.68	0.021
		First area entered for 30 sec	0.46	11.92	0.008
		Last entered area	0.44	9.84	0.020
(*E*)-β-Farnesene[a]	Female	First entered area	0.50	16.72	0.001
		First area entered for 30 sec	0.52	16.60	<0.001
		Last entered area	0.54	22.80	<0.001
	Male	First entered area	0.52	19.60	<0.001
		First area entered for 30 sec	0.54	22.48	<0.001
		Last entered area	0.58	29.20	<0.001

[a] Natural source of EβF was 20 unwinged adult aphids, *M. persicae* Sultzer (crushed in the olfactometer release chamber)

themselves toward aphid prey by discriminating aphid punctual and EβF emission from the continuous plant release of EβF, usually joined with (−)-β-caryophyllene emission (Dawson et al. 1984). The latter has also been shown previously to counteract the attraction of lady beetles to EβF, acting thus as a natural alarm pheromone inhibitor, allowing the beetles to differentiate between EβF of plant and aphid origin. As *H. axyridis* releases its aggregation pheromone at the end of autumn, when the temperatures get lower and aphids are present in lower densities, no inhibition happens.

Our results demonstrated that (−)-β-caryophyllene and EβF act as semiochemicals for *H. axyridis*. Indeed, (1) there are some specific neuronal receptors that allow their perception and (2) a behavioral attraction/aggregation was noted in the two behavioral assays. Al Abassi et al. (2000) obtained similar electrophysiological results while studying *C. septempunctata* olfactory cell responses toward the same two sesquiterpenes: Cells having high specificity for (−)-β-caryophyllene and EβF were identified. In the 12-spotted lady beetle, *Coleomegilla maculata* (Coleoptera: Coccinellidae), the highest EAG responses were obtained while testing EβF, (−)-β-caryophyllene, and two other terpenoid alcohols (Zhu et al. 1999). Therefore, it is not surprising that *H. axyridis*, which also belongs to the Coccinellidae, possesses similar specific receptors.

(−)-β-Caryophyllene is specifically released by *H. axyridis* females in overwintering aggregation conditions (Brown et al. 2006). The production of (−)-β-caryophyllene by females of *H. axyridis* was confirmed in the present study by volatile collection and subsequent GC-MS analysis (data not shown). Whereas cases of aggregation pheromone release by one sex only are common in the Coleoptera (e.g., Verheggen et al. 2007), the use of a chemical commonly found in the headspace of various plants as an aggregation pheromone might be inappropriate. One might put forth the assumption that lady beetles have different antennal sensitivity to (−)-β-caryophyllene according to the season, being more sensitive during winter conditions. (−)-β-Caryophyllene has an attractive effect on *H.*

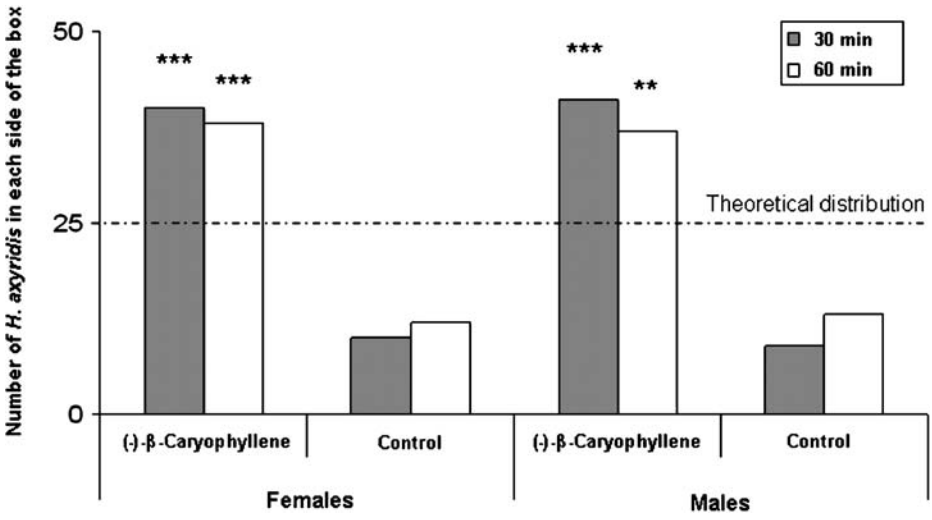


Fig. 1 Observed distribution of female and male *H. axyridis* (N=50 for each sex) relative to the release site of (−)-β-caryophyllene in an aggregation bioassay. *Double* and *triple asterisks* indicate significant differences between observed and expected distributions at $P<0.01$ and $P<0.001$, respectively (χ^2 goodness-of-fit test)

axyridis, although the responses of the females in the four-arm olfactometer were significant in only one of the three behavioral observations. However, in the second set of behavioral assays, the distribution of both males and females in the (−)-β-caryophyllene-treated side of the box was significant. These behavioral data, the antennal perception of (−)-β-caryophyllene, and its specific period of release (i.e., in the early winter) support the hypothesis that it acts as a component of an aggregation pheromone rather than a sex pheromone.

Like other lady beetle species, including *A. bipunctata* (Hemptinne et al. 2000; Francis et al. 2004), *H. convergens* (Acar et al. 2001), and *C. septempunctata* (Nakamuta 1991; Al Abassi et al. 2000), *H. axyridis* is able to perceive and orient itself toward EβF, the main component of the aphid alarm pheromone. Our electrophysiological and behavioral results support previous research that demonstrated that *H. axyridis* has a high ability to track aphid populations in space and time (Osawa 2000). Indeed, this aphid predator showed fast and pronounced orientation behavior toward the EβF source in the four-arm olfactometer. Having the ability to localize aphids under predation represents an undeniable advantage for a mobile and voracious predator like *H. axyridis*. Although previous studies demonstrated that *H. axyridis* responded to volatiles from aphids and aphid-damaged plants (Han and Chen 2002), our results do not support previous work that demonstrated that *H. axyridis* was attracted toward a colony of *A. pisum*, but not toward cornicle secretions (Mondor and Roitberg 2000), which are known to contain EβF (Francis et al. 2005).

In summary, push–pull strategies that target the use of *H. axyridis* as a biological control agent in aphid-infested sites, or that are designed to control this new urban pest in human dwellings, should take into account the potential attractive effect that (−)-β-caryophyllene could have in outdoor conditions. Our data also suggest that, in aphid biological control strategies, one should incorporate EβF as an attractant for various aphid natural enemies, including *H. axyridis*.

Acknowledgments The authors are grateful to Dr. Bartram, from Max Planck Institute for Chemical Ecology, for the synthesis of (*E*)-β-farnesene. We also thank Dr. Yves Brostaux and Adeline Gillet, from Gembloux Agricultural University, for advice on statistical analysis. This work was supported by the *Fonds National pour la Recherche Scientifique* (grant M 2.4.586.04.F).

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visibly attractive click beetles of Central Europe is a rare species throughout its entire distribution, which ranges from Spain to the Caucasus, and from Italy in the south to Sweden in the north (Horion 1953).

Elater ferrugineus develops in hollows of various old living deciduous trees that are filled with a special kind of wood mold, which is usually generated by the larvae of scarab beetle species such as *Osmoderma eremita* (Scopoli), *Gnorimus variabilis* (L.), *Protaetia aeruginosa* (Drury), or *Protaetia lugubris* (Herbst). The larvae of *E. ferrugineus* are facultatively carnivorous and occasionally prey on eggs and larvae of these scarab beetles. However, they can also develop on wood mold alone. The life cycle of *E. ferrugineus* can last up to 6 years, depending on the abundance of prey organisms in the breeding substrate. Unless an inhabited tree is dying or felled, it can sustain a population of *E. ferrugineus* for several decades.

In Central Europe, adults of *E. ferrugineus* occur in midsummer from late June to August. However, the adults live cryptically and are rarely encountered in the field. This, and occasional reports on single specimens attracted by artificial light sources, have led several authors to conclude that *E. ferrugineus* might be nocturnally active (e.g., Horion 1953; Lohse 1979). Larvae of *E. ferrugineus* have been recorded more frequently, as they are present throughout the year much more abundantly than the adults and strictly located in their breeding substrate. As a result, adult *E. ferrugineus* preserved in collections have mostly been bred from late instars. Nevertheless, larvae are normally only collected when an inhabited old tree is occasionally felled or otherwise opened, which usually accompanies the destruction of the habitat.

As a result of massive habitat loss in Europe caused by decades of intense and profit-orientated forestry, *E. ferrugineus* appears in the Red Data Books throughout its distribution, classified as endangered (e.g., Germany: Geiser 1998; Sweden: Gärdenfors 2005) or critically endangered (e.g., Czech Republic: Vavrá 2005). In Central Europe, *E. ferrugineus* is regarded as an "Urwald relict species," i.e., a species associated with primeval forest ("Urwald") structures and features (Müller et al. 2005), and is used as indicator species for old and species-rich deciduous forests in landscape ecology (Schmidl and Bussler 2004). However, a large degree of experience is needed for recognizing trees suitable for *E. ferrugineus* and for distinguishing larvae from even many similar more abundant species. Therefore, especially in official ecological surveys, the species is frequently overlooked.

In this paper, we report the identification and synthesis of the sex pheromone of *E. ferrugineus*. In addition to chemotaxonomical aspects, we were interested in developing a specific and effective monitoring tool, and we found the synthetic pheromone blend to be highly attractive to swarming *E. ferrugineus* males.

Methods and Materials

Laboratory Studies

Insects Late instars of *E. ferrugineus* (>4 cm) were collected from three different field sites in Germany: 1) Forst Göhrde/Niedersachsen, seven larvae from an old hollow oak, *Quercus petraea* (Matt.) Liebl. (Jan. 2002, March 2003); 2) Kühkopf/Hessen, three larvae from a wind-broken hollow ash, *Fraxinus excelsior* L. (Feb. 2005); and 3) Tübingen/Baden-Württemberg, eight larvae from an old linden, *Tilia* sp. (March 2005).

Larvae were kept isolated at room temperature in single plastic screw-capped glass jars (500 ml) with the wood mold that they were collected in. They were fed with pieces of

yellow mealworm, *Tenebrio molitor* L., during the following months. Pupation took place in early May of the collection year, and adult beetles emerged about 3 wk later. In total, seven females were reared and examined. Sexes of the beetles were distinguished by examining the shape of their antennae, which are considerably longer and more serrate in the males. They were sometimes verified by examination of the genitalia.

Extracts Beetles were deep-frozen (20 min at −20°C), the abdomen was broken off by bending it over to the ventral side, and the tergites were removed to excise the paired pheromone glands that are similar to those described by Merivee and Erm (1993) for the click beetle *Agriotes obscurus*. Separate extracts were prepared for each beetle by transferring the glands to 300 µl CH_2Cl_2.

Chemical Analyses Analyses of extracts were carried out by coupled gas chromatography–mass spectrometry (GC–MS). A 6890 GC/5973N MSD quadrupole instrument (Agilent Technologies, Santa Clara, CA, USA) was employed in electron impact mode at 70 eV, 230°C. Compounds were separated with a 30 m×0.25 mm i.d., 0.25 µm film thickness HP5-MS fused silica capillary column (Agilent), starting at 60°C for 3 min, then programmed to 300°C at a rate of 3°C/min. Carrier gas was helium with a flow rate of 1.2 ml/min. In addition, a 50 m×0.25 mm i.d., 0.27-µm film thickness FS-FFAP column (Macherey & Nagel, Düren, Germany) was used (60°C for 3 min, then 3°C/min to 220°C, and held for 20 min).

For separation of vicinal dimethyl thioderivatives of extract compounds, produced for the determination of double bond positions, the HP5-MS capillary column was used as described, but the final temperature of 300°C was held for 60 min. Reaction of double bonds was carried out with dimethyl disulfide (DMDS) (Buser et al. 1983). Nuclear magnetic resonance (NMR) spectra of synthetic compounds were recorded with a Varian INOVA 300 instrument (Varian, Palo Alto, CA, USA).

Chemical Syntheses Chemicals and solvents were purchased from Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany) and were of highest purity available. Purification of synthetic products was carried out by flash chromatography on silica gel (silica 32-63, 60 Å ICN-Biomedicals, Eschwege, Germany) at 1.3 bar with mixtures of ethyl acetate and hexane.

Monobenzylation of commercially available 1,5-pentanediol (Sigma-Aldrich) employing sodium hydride and benzyl bromide gave alcohol **1**, which was oxidized to the corresponding aldehyde **2** by Swern oxidation (Fig. 1). Wittig reaction of **2** with isobutyltriphenylphosphonium bromide produced the benzyl ether of 7-methyl-5-octen-1-ol (**3**), which was hydrogenated over 10% Pd/C catalyst to yield 7-methyloctan-1-ol (**4**), the alcohol moiety of all subsequently synthesized esters. An aliquot of **4** was oxidized to 7-methyl octanoic acid (**5**) by using CrO_3/H_2SO_4 in acetone (Jones chromic acid). 7-Methyloctyl octanoate (**6**) was synthesized from **4** and octanoyl chloride (Sigma-Aldrich) in ether. Oxalyl chloride was used to transform acid **5** into its chloride, which was treated *in situ* with **4** to yield 7-methyloctyl 7-methyloctanoate (**7**). With the same procedure, commercially available 5-methylhexanol (**8**) was converted to the acid **9** and esterified with **4** to yield 7-methyloctyl 5-methylhexanoate (**10**). Similarly, (*Z*)-4-decenoic acid was synthesized from (*Z*)-4-decenol (**11**), and converted to 7-methyloctyl (*Z*)-4-decenoate (**12**) with alcohol **4**.

The synthesis provided alcohol **4** in 34 % from **1**, the esters **6**, **7**, **10**, and **12** in 80–85% from alcohol **4** (Fig. 1). Chemical purity of the four esters after chromatographic purification was >98%.

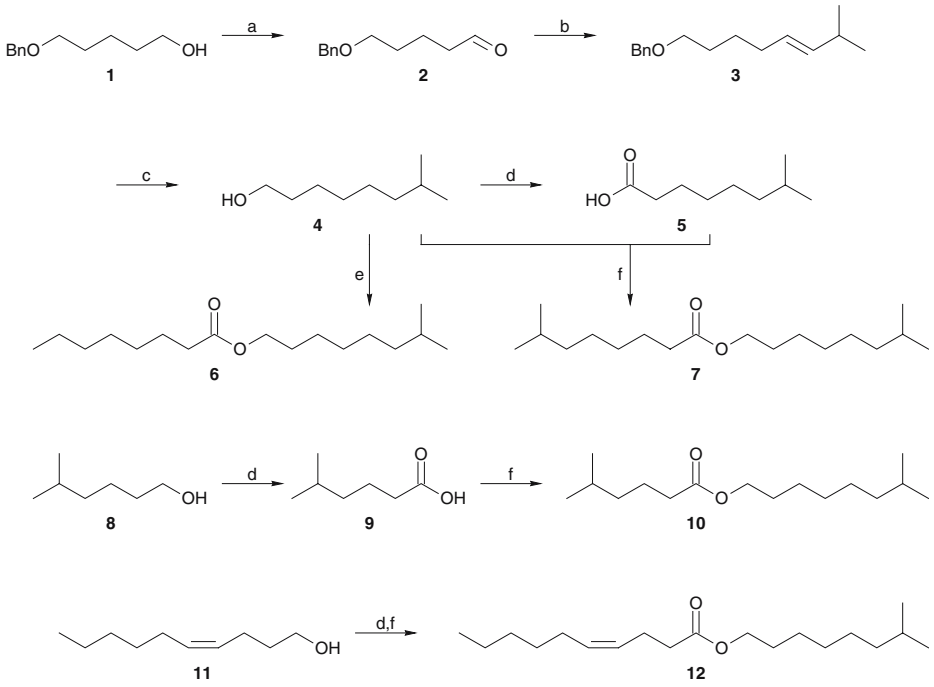


Fig. 1 a) $(COCl)_2$/DMSO/Et_3N, CH_2Cl_2; b) $Ph_3PCH_2CH(CH_3)_2Br$, BuLi/THF; c) H_2, Pd/C; d) Jones CrO_3, acetone; e) Pyridine/Et_2O, $H_3C(CH_2)_6COCl$; f) $(COCl)_2/CH_2Cl_2$, 4/pyridine

Spectroscopic Data

7-Methyloctan-1-ol (4) ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm)=0.86 (d, 6H, J=6.6 Hz, 7-CH_3), 1.12–1.20 (m, 2H, 6-H), 1.24–1.37 (m, 6H), 1.38 (br. s, 1H, OH), 1.44–1.61 (m, 3H, 2-H/ 7-H), 3.63 (t, 2H, J=6.4 Hz, 1-H).

^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm)=22.64 (2q, 7-CH_3), 27.95 (d, 7-C), 25.77/ 27.34/ 29.68/ 32.82/ 38.95 (5t, 2-C/ 3-C/ 4-C/ 5-C/ 6-C), 63.10 (t, 1-C).

MS (70 eV): m/z (%) = 126 (0.2) [M^+-H_2O], 112 (1), 111 (7), 98 (5), 97 (2), 85 (2), 84 (10), 83 (26), 82 (11), 71 (3), 70 (26), 69 (57), 68 (8), 67 (6), 58 (2), 57 (40), 56 (100), 55 (57), 54 (3), 53 (3), 45 (2), 44 (3), 43 (53), 42 (18), 41 (49), 40 (3), 39 (11).

7-Methyloctyl octanoate (6) ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.86 (d, 6H, J= 6.6 Hz, 7′-CH_3), 0.88 (t, 3H, J = 7.7 Hz, 8-H), 1.12–1.20 (m, 2H, 6′-H), 1.21–1.38 (m, 14H), 1.47–1.68 (m, 5H, 5-H/ 2′-H/ 7′-H), 2.29 (t, 2H, J=7.7 Hz, 2-H), 4.05 (t, 2H, J= 6.4 Hz, 1-H).

^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 14.04 (q, 8-C), 22.59 (t, 7-C), 22.61 (2q, 7′-CH_3), 25.03 (t, 3-C), 27.93 (d, 7′-C), 25.96/ 27.26/ 28.66/ 28.92/ 29.11/ 29.50 (6t, 4-C/ 5-C/ 2′-C/ 3′-C/ 4′-C/ 5′-C), 31.66 (t, 6-C), 34.41 (t, 2-C), 38.91 (t, 6′-C), 64.38 (t, 1′-C), 173.99 (s, 1′-C).

MS (70 eV): m/z (%) = 270 (0.1) [M^+], 171 (3), 146 (8), 145 (91), 144 (8), 128 (4), 127 (39), 126 (22), 125 (2), 116 (3), 115 (3), 112 (3), 111 (30), 109 (3), 101 (8), 99 (2), 98 (22), 97 (7), 89 (5), 87 (4), 85 (13), 84 (24), 83 (26), 82 (14), 81 (3), 73 (9), 71 (23), 70 (26), 69 (46), 68 (9), 67 (7), 61 (10), 60 (7), 58 (4), 57 (100), 56 (80), 55 (49), 54 (4), 53 (3), 44 (2), 43 (71), 42 (16), 41 (49), 39 (6).

7-Methyloctyl 7-methyloctanoate (7) ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.86 (d, 12H, J=6.6 Hz, 7-CH_3 and 7′-CH_3), 1.11–1.21 (m, 4H, 6-H and 6′-H), 1.22–1.38 (m, 10H, 4-H/ 5-H/ 3′-H/ 4′-H/ 5′-H), 1.46–1.57 (m, 2H, 7-H and 7′-H), 1.58–1.68 (m, 4H, 3-H and 2′-H), 2.29 (t, 2H, J=7.4 Hz, 2-H), 4.05 (t, 2H, J=6.8 Hz, 1′-H).

^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 22.60/ 22.62 (4q, 7-CH_3 and 7′-CH_3), 25.07 (t, 3-C), 27.91/ 27.94 (2d, 7-C and 7′-C), 25.98/ 27.02/ 27.26/ 28.67/ 29.41/ 29.51 (6t, 4-C/ 5-C/ 2′-C/ 3′-C/ 4′-C/ 5′-C), 34.42 (t, 2-C), 38.80/ 38.91 (2t, 6-C and 6′-C), 64.40 (t, 1′-C), 174.00 (s, 1-C).

MS (70 eV): m/z (%) = 284 (0.3) [M^+], 171 (3), 160 (8), 159 (77), 158 (4), 143 (5), 141 (10), 140 (3), 139 (4), 127 (4), 126 (24), 125 (7), 123 (18), 122 (3), 116 (3), 115 (8), 112 (4), 111 (33), 103 (3), 101 (3), 99 (4), 98 (25), 97 (16), 96 (3), 95 (3), 87 (4), 85 (19), 84 (24), 83 (27), 82 (17), 81 (15), 73 (13), 72 (3), 71 (39), 70 (28), 69 (57), 68 (9), 67 (13), 61 (12), 60 (7), 59 (3), 58 (5), 57 (100), 56 (94), 55 (69), 54 (5) 53 (4), 44 (4), 43 (99), 42 (18), 41 (59), 39 (7).

*7-Methyloctyl 5-methylhexanoate (**10**)* ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.86 (d, 6H, J=6.6 Hz, 7′-CH_3), 0.88 (d, 6H, J=6.6 Hz, 5-CH_3), 1.11–1.22 (m, 4H, 4-H and 6′-H), 1.23–1.37 (m, 6H, 3′-H/ 4′-H/ 5′-H), 1.46–1.68 (m, 6H, 3-H/ 5-H/ 2′-H/ 7′-H), 2.27 (t, 2H, J=7.7 Hz, 2-H), 4.05 (t, 2H, J=6.8 Hz, 1′-H).

^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 22.46 (2q, 5-CH_3), 22.61 (2q, 7′-CH_3), 22.90 (t, 3-C), 27.76 (d, 5-C), 27.92 (d, 7′-C), 25.96/ 27.25/ 28.66/ 29.49 (4t, 2′-C/ 3′-C/ 4′-C/ 5′-C), 34.61 (t, 2-C), 38.38 (t, 4-C), 38.90 (t, 6′-C), 64.38 (t, 1′-C), 173.96 (s, 1-C).

MS (70 eV): m/z (%) = 213 (1) [M^+-C_3H_7], 171 (3), 132 (5), 131 (62), 130 (2), 129 (2), 127 (2), 126 (16), 116 (3), 115 (9), 113 (15), 112 (4), 111 (28), 98 (17), 97 (10), 96 (5), 95 (58), 94 (2), 89 (4), 87 (17), 85 (15), 84 (19), 83 (22), 82 (13), 81 (2), 73 (6), 71 (26), 70 (27), 69 (66), 68 (9), 67 (7), 61 (9), 60 (7), 58 (4), 57 (73), 56 (95), 55 (51), 54 (4), 53 (4), 44 (4), 43 (100), 42 (20), 41 (60), 40 (2), 39 (8).

*7-Methyloctyl (Z)-4-decenoate (**12**)* ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 0.86 (d, 6H, J=6.6 Hz, 7′-CH_3), 0.88 (t, 3H, J=7.7 Hz, 10-H), 1.11–1.21 (m, 2H, 6′-H), 1.22–1.40 (m, 12H, 7-H/ 8-H/ 9-H/ 3′-H/ 4′-H/ 5′-H), 1.46–1.67 (m, 3H, 2′-H and 7′-H), 1.99–2.08 (m, 1H, 6-H), 2.27–2.38 (m, 4H, 2-H and 3-H), 4.06 (t, 2H, J=6.9 Hz, 1′-H), 5.27–5.47 (m, 2H, 4-H and 5-H).

^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) = 14.05 (q, 10-C), 22.62 (2q, 7′-CH_3), 22.56/ 22.87 (2t, 3-C and 9-C), 27.93 (d, 7′-C), 25.95/ 27.15/ 27.26/ 28.64/ 29.30/ 29.51 (6t, 6-C/ 7-C/ 2′-C/ 3′-C/ 4′-C/ 5′-C), 31.49 (t, 8-C), 34.46 (t, 2-C), 38.91 (t, 6′-C), 64.53 (t, 1′-C), 127.34 (d, 4-C), 131.51 (d, 5-C), 173.36 (s, 1-C).

MS (70 eV): m/z (%) = 296 (1) [M^+], 171 (12), 170 (2), 153 (16), 152 (60), 151 (5), 141 (3), 137 (3), 135 (11), 134 (9), 133 (5), 128 (2), 127 (9), 126 (4), 124 (13), 123 (18), 115 (3), 114 (12), 113 (9), 112 (3), 111 (26), 110 (64), 109 (20), 108 (11), 107 (4), 101 (7), 100 (7), 99 (4), 98 (15), 97 (19), 96 (51), 95 (12), 94 (4), 93 (3), 87 (4), 86 (4), 85 (56), 84 (63), 83 (27), 82 (17), 81 (23), 80 (3), 79 (7), 73 (6), 72 (5), 71 (96), 70 (17), 69 (75), 68 (21), 67 (30), 65 (3), 61 (3), 60 (4), 58 (5), 57 (100), 56 (37), 55 (90), 54 (24), 53 (11), 44 (4), 43 (98), 42 (15), 41 (74), 40 (3), 39 (11).

In the NMR data of the esters **6**, **7**, **10**, and **12**, carbon atom numbers of the acid moieties are indicated by an apostrophe.

Field Studies

Bait Dispensers and Traps A synthetic blend of 7-methyloctyl 5-methylhexanoate (**10**), 7-methyloctyl octanoate (**6**), 7-methyloctyl 7-methyloctanoate (**7**), and 7-methyloctyl (*Z*)-4-

decenoate (**12**) was mixed in a ratio of **10**:**6**:**7**:**12** = 1:1:3:3, which resembled the natural ratio found in the glands. Dispensers were prepared from 0.2-ml PCR tubes (Thermo-Tube™, Peqlab, Erlangen, Germany) by piercing each tube with an insect pin (diameter 0.5 mm) on the front side 2 mm below the lid (Fig. 2), and then adding 2 mg of the four-compound blend into each tube. Under laboratory conditions (ca. 24°C), the odor dispensers had a stable mean release rate of approximately 10 μg per day, regardless of the amount in the tubes (unpublished data).

Funnel traps (Fig. 2) were modified from those described by Ruther et al. (2000). Two transparent plexiglass sheets (17 cm height × 20 cm width) were arranged crosswise over a 185-mm powder funnel with its outlet (diam. 40 mm) connected to a 1-l polyethylene collection bottle. Dispensers were mounted on a thread in a gap at the top between the plastic sheets (Fig. 2). Each collecting bottle was filled with brine (200 ml) to preserve the beetles and minimize the possible attraction of beetles to the already captured individuals.

Flight Bioassays Bioassays were performed from 14 July to 5 August, 2005 in three urban parks in Tübingen, Stuttgart, and Hohenheim (Baden-Württemberg, Southwest Germany). Predominant old deciduous trees at the study sites were *Tilia* sp. (Tübingen), *Fagus sylvatica* L., *Platanus* × *hybrida* Br. (Stuttgart), and *Populus nigra* L. (Hohenheim), with some individuals up to 300 years old. A total of 28 traps were grouped into 14 sets, each set containing a baited trap and an unbaited control. Four of these sets were used in Tübingen, five in Stuttgart, and five in Hohenheim. Traps were suspended approximately 5–10 m above the ground from the lower crown branches of solitary old trees. The distance between traps within one set was about 10 m, whereas the distances between the sets at a site were at least 50 m. Traps were checked weekly, captured beetles were removed, and the brine was replaced. After sex determination, numbers of beetles trapped were analyzed by the Wilcoxon matched-pairs test using Statistica 6.1 (Statsoft 2003).

An additional trap was placed 15 m above the ground in front of the lab window (Hohenheim Institute, fourth floor) to monitor the daily flight activity. This trap was observed continuously during the day, arriving beetles were removed immediately, and time and sex were noted. As this trap was without an empty control, catches were not analyzed statistically.

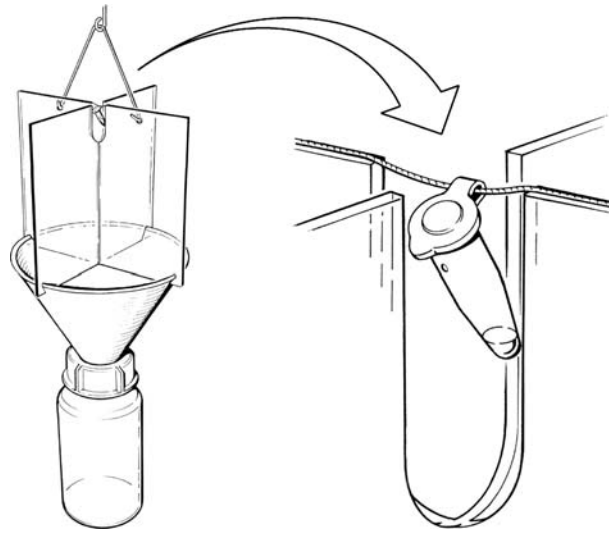


Fig. 2 Funnel trap used for capturing *Elater ferrugineus* and closeup of the mounted pheromone dispenser

Results

Laboratory Studies

Gas chromatography–mass spectrometry (GC–MS) analysis of the gland extracts revealed four main compounds (**A**–**D**, Fig. 3) with only small quantitative variation among samples of different females. Compounds **A**–**C** showed typical mass spectra of long-chained saturated esters with 16, 17, and 18 carbon atoms, respectively, nine of these belonging to the alcohol moiety. The difference in retention time between **A** and **B** was more than twice the difference between **B** and **C** (Fig. 3), suggesting a methyl-branched chain for compound **A**.

A micro-transesterification performed with half of a crude extract by substituting the dichloromethane with an equal amount of methanol and storing the extract at 60°C for 48 hr yielded only one alcohol. It showed a shorter retention time than *n*-nonanol and was identified as 7-methyloctanol (**4**) by comparison with an authentic sample. Compound **B** was subsequently identified as 7-methyloctyl octanoate (**6**) by converting synthetic **4** to its heptanoate, octanoate, and nonanoate by employing the corresponding *n*-acyl chlorides and comparing retention times and spectra. Compound **A**, showing a shorter retention time than the *n*-heptanoate of **4** and indicating another methyl branch, turned out to be 7-methyloctyl 5-methylhexanoate (**10**). Accordingly, compound **C** was identified as 7-methyloctyl 7-methyloctanoate (**7**).

The mass spectrum of extract compound **D** resembled those of **A**, **B**, and **C**, but a molecular mass of 296 g/mol indicated a double bond. Given that transesterification only produced **4**, the double bond could be located only in the acid moiety of **D**, which pointed to a 7-methyloctyl decenoate. DMDS-derivatization yielded a product with a molecular ion of *m*/*z* 390 and a pair of diagnostic ions of *m*/*z* 131 and 259, indicating that the unsaturation was between C-4 and C-5 of the acid moiety (Fig. 4).

For determination of the double bond geometry, 7-methyloctyl (*Z*)-4-decenoate (**12**) was synthesized from commercially available (*Z*)-4-decenol (**11**). The mass spectrum of **12** and the retention times on the HP5-MS and FS-FFAP capillary columns were identical to those of extract compound **D** (data not shown). Thus, we concluded that the natural product has

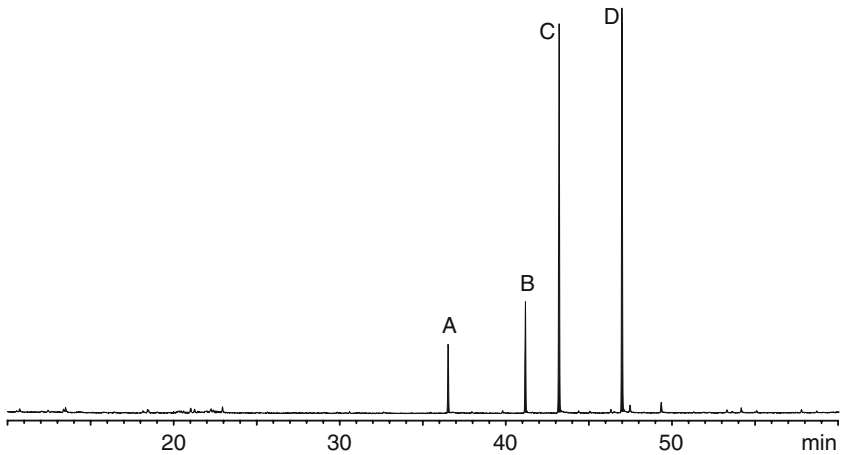


Fig. 3 Representative gas chromatogram (total ion current) from an analysis of a female *Elater ferrugineus* gland extract (30 m HP5-MS capillary column, 3 min at 60°C, then 3°C/min to 300°C)

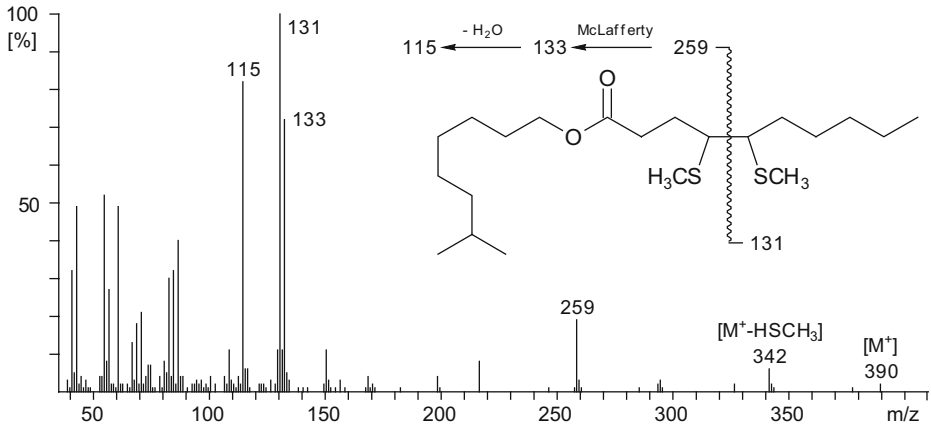


Fig. 4 Determination of double bond position in compound **D**: mass spectrum of the DMDS derivative and assignment of prominent ions to the resulting structure

the (*Z*)-configuration. In two samples, the amount of the major pheromone component **D** was estimated to be 6.9 and 5.9 μg/female, respectively.

Field Studies

Unexpectedly, the first proof for the attractiveness of the identified compounds was the response of several male *E. ferrugineus* that appeared at the open lab window (fourth floor) while the substances were purified over SiO_2 (12 July, 2005). In the field bioassay, a total of 57 male *E. ferrugineus* were caught with the pheromone blend, and one beetle was caught in an unbaited control trap, showing a highly significant difference (Fig. 5). As a result of single traps that vanished during the test, the number of replicates were reduced from 42 to 39.

An additional 33 males were caught at the lab window, including the catches in the funnel trap. Most responses were observed directly, or at least recorded within an hour of the response. Catches occurred exclusively during the day. The earliest recorded response was at 7:25 (CET) and the latest was at 19:30. Within this range, no preferred time of the day was found, but warm and sultry weather conditions, without intense sunlight and wind, seemed to be essential. In 2005, the flight season ended during the first week of August.

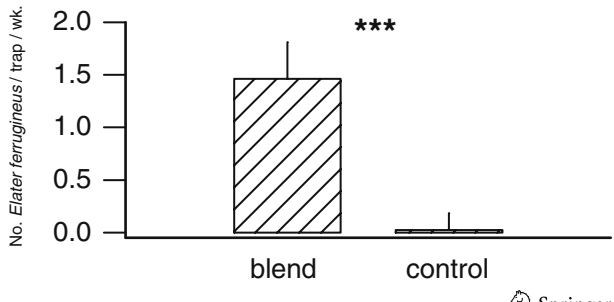


Fig. 5 Mean (+ SE) weekly trap catches of *Elater ferrugineus* from 14 July to 5 August 2005 in response to the synthetic blend of compounds 6,7,10, and 12 (Fig. 1) (***$P<0.001$, $N=39$, Wilcoxon matched pairs test)

Discussion

Our results show that the identified gland compounds comprise the female sex pheromone of *E. ferrugineus*. Nevertheless, it remains unknown whether all four compounds are necessary to attract the males, or if only one or a few of them are biologically active. The benefits of further field testing of all combinations of these four compounds, however, must be weighed against the potential damage that trapping would do to populations of this rare beetle.

Although the attractive effect of our four-compound-blend was highly significant, the total number of captured males in the traps was relatively low. However, *E. ferrugineus* is a rare species that likely occurs at a much lower population density than the average pest insect. In addition, field tests in 2005 were conducted during the second half of the flight period because it took longer than expected to complete the syntheses to bait the experimental traps. In 2006, when we set out nonkilling traps to survey and estimate population sizes, we recorded daily trap catches of up to 20 males/trap during the first half of the flight season (unpublished data).

Although their structures are comparatively simple, all compounds represent new natural products. The *iso*-methyl branches in the acid or alcohol moieties likely result from a biosynthesis that employs leucine as the chain initiating unit, which is then, after conversion to an isovaleryl derivative, elongated with acetyl units. A similar biosynthetic pathway has been found by Charlton and Roelofs (1991) in an arctiid moth.

Before our study, female-produced click beetle pheromones could be divided into three groups based on their structural properties. Interestingly, this division fits the taxonomical classification as well. The first group comprises the members of the genus *Agriotes* (Elaterinae: Agriotini), from which, as a result of their economical importance, comparatively many species have been investigated (e.g., Tóth et al. 2003). In females of this genus, several esters of acyclic isoprenoid alcohols with equal chain acids (two to eight carbon atoms) have been identified as pheromone components. A typical example is (*E*,*E*)-farnesyl butyrate, which, together with geranyl butyrate, is the major component of the sex pheromone of *A. brevis* Candeze (Tóth et al. 2002). In a second group, which contains the genus *Melanotus* (Melanotinae), compounds resembling moth pheromones have been reported, i.e., unbranched esters of alcohols with 12 and 14 carbon atoms. The eastern Palearctic *Melanotus okinawensis* Ohira uses dodecyl acetate, whereas in *M. sakishimensis* Ohira, a mixture of (*E*9,11)-dodecadienyl butyrate and -hexanoate is present (Iwanaga and Kawamura 2000). The central European species, *M. punctolineatus* (Pelerin), uses a tetradecenyl butyrate, whereas *M. rufipes* (Herbst) uses a tetradecadienyl butyrate (Tolasch, unpublished). A third group contains the first biologically active and most simple compounds identified from click beetles. These are hexanoic acid and pentanoic acid from *Limonius canus* LeConte and *L. californicus* (Mannerheim), respectively (Jacobson et al. 1968), both members of the subfamily Dendrometrinae.

The four 7-methyloctyl esters reported in this paper do not fit well into any of these three groups. However, *E. ferrugineus* (Elaterinae: Elaterini) does not belong to any of the subfamilies or tribes noted in the taxonomic discussion above. Further investigations of other members of the Elaterini (e.g., *Sericus* spp.) would be instructive to elucidate whether this tribe represents a fourth group with respect to the structural properties of their sex pheromones.

In our study, males of *E. ferrugineus* were caught only during the day. This is remarkable, as *E. ferrugineus* has generally been considered to be a nocturnal species. One of us (T. T.) questioned this hypothesis because he observed an impressive midday swarming flight of

E. ferrugineus (28 July 1985), when several beetles were hovering in the canopy of some old willow trees, *Salix* spp., near the Elb River in Niedersachsen at 13.00 (CET). No such swarming behavior has been reported in the literature to date, and discussions with several coleopterists suggested that this observation was quite unique. However, single beetles attracted occasionally by artificial light sources during the night may have been dispersing females.

The hermit beetle, *Osmoderma eremita* (Scopoli), is one of the scarab beetles that inhabit and extend hollows in old living deciduous trees, thus making them suitable for *E. ferrugineus*. The peach-like scent of *O. eremita* was recently identified as (*R*)-γ-decalactone (Larsson et al. 2003), which is emitted in large quantities by the males as a pheromone that indicates suitable tree cavities to conspecifics. Interestingly, Svensson et al. (2004) found (*R*)-γ-decalactone to be slightly attractive to *E. ferrugineus* as well. While trapping *O. eremita* with lactone-baited funnel traps suspended near inhabited tree hollows, a few male and female *E. ferrugineus* were obtained as by-catches. The authors suggested a kairomonal role of the substance that leads *E. ferrugineus* to suitable tree hollows, and they discussed a possible function for *E. ferrugineus* males as an "indirect cue to find females of their own species." However, because our study reveals the existence of a highly attractive sex pheromone, the rationale for the use of (*R*)-γ-decalactone as a sexual kairomone appears moot, particularly as *E. ferrugineus* frequently develops in the absence of *O. eremita*.

With respect to conservation biology, the present study highlights the large potential of sex pheromones for monitoring populations of scarce and cryptic species. Insects that inhabit old hollow trees are usually collected by direct sampling of the wood mold, by placing pitfall traps into the cavities, or by suspending window traps outside the hollows (e.g., Ranius and Jansson 2002). Adults of *Elater ferrugineus*, however, are rarely caught with these methods, especially as, in the majority of cases, the openings of the inhabited cavities are narrow and located in the treetop, and inaccessible for mold sampling or pitfall traps.

In contrast, our four-ester blend provides a monitoring tool for *E. ferrugineus*. The serendipitous responses at our laboratory window suggest that traps do not have to be placed in the direct vicinity of inhabited trees, and one or two traps would be sufficient to cover a large area. Males are trapped exclusively; ovipositing females are preserved; and no other species (e.g., the highly protected *O. eremita*) are harmed. For entomofaunistic studies, pheromone traps will be particularly useful in regions where *E. ferrugineus* is considered extinct, but perhaps still exists below the limit of detection offered by conventional collecting methods. For example, the population that we identified that inhabits the old park area around the University of Hohenheim was unknown before our pheromone study. Generations of biology students had conducted their field studies at this site that included substantial insect collecting. Apart from our recent catches, only eight specimens of *E. ferrugineus* had been recorded during the last 60 years in the entire federal district of Baden-Württemberg/Germany (Frank and Konzelmann 2002), consisting of seven larval records and a single adult record.

Acknowledgments Financial support of this work by the Geschwister-Stauder-Stiftung, Hohenheim, is gratefully acknowledged. We thank Bernd Schmid for drawing the field trap illustration.

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the larvae of the coccinellid *Adalia bipunctata* (L.) that showed ODP activity. They identified the constituents to be a complex mixture of typical insect cuticular hydrocarbons but did not test for the presence of compounds not detectable by gas chromatography (GC)/mass spectrometry (MS). Furthermore, no bioassays were performed with individual compounds. We report here the identification of an ODP present in extracts of first instars of *C. sexmaculata*, from bioassay-guided fractionation of the extract.

Materials and Methods

Analysis The procedures in GC/MS are as follows: HP 5890 GC coupled with HP 5973 mass selective detector, splitless injection, BPX-5 column (SGE), 60–320°C, 5°C/min. In electrospray ionization (ESI)-MS, analyses were performed on a Finnigan MAT 95 XLT with direct injection by using a microspray interface in positive and negative ionization mode. Samples were dissolved in a 1:1 CH_2Cl_2/CH_3OH mixture. Dimethyldisulfide (DMDS) derivatives were prepared according to Schulz et al. (2000). In nuclear magnetic resonance (NMR) experiments, a DRX-400 (400 MHz, Bruker) was used. Precocinelline and hippocasine were identified by comparison with respective mass spectra in the National Institute of Standards and Technology database. Injection of coccinelline into the injection port of a gas chromatograph at 250°C furnished a chromatogram that contained these two compounds as the only major peaks.

Separation Unfed first instars (5,000, laboratory culture, generations 5–20 originating from the Arab Emirates, 2004) were extracted with 20 ml chloroform for 10 min. The extract was concentrated under a gentle stream of N_2, separated on Sephadex LH-20 (12×1.4 cm) with dichloromethane/methanol (1:1) as eluent into 35 fractions. Fractions that showed an almost identical compound profile by GC/MS were combined, resulting in six fractions, which were analyzed by GC/MS and ESI-MS. After concentration, residues were redissolved in 5 ml of dichloromethane/chloroform (1:4) for bioassays. The final concentration was 135 larval equivalents/ml, assuming that no dilution by the separation process occurred.

Bioassays Oviposition-deterring effects were studied in dual-choice tests as described (Růžička 1997). The test solution (three different concentrations, see Table 1) or chloroform as control was applied to filter papers in dual-choice tests. Ten females of *C. sexmaculata* were placed in the test chamber for 24 h. Water and surfeit of *Aphis fabae* Scopoli were provided as food. Each test had ten replicates. The numbers of eggs laid on treated and

Table 1 Number of eggs laid by *Cheilomenes sexmaculata* females on filter papers treated with a chloroform extract of conspecific first-instar larvae (*) and with different amounts of (*Z*)-pentacos-12-ene[a] vs solvent controls

Concentration μg/cm^2 or larval equivalent/cm^2 (*)	Treatment	Control	Wilcoxon test
0.25*	46±6.7	81.5±4.7	P=0.002
1.25	121.5±12.4	207.5±10.9	P=0.004
0.125	105.0±16.3	166.7±11.0	P=0.006
0.0125	148.7±10.4	135.9±10.4	P=0.625

[a] Mean number of eggs laid in dual-choice tests with ten replicates of ten females. Altogether one hundred individuals were tested in ten replicates in one test. Wilcoxon paired sample test.

control papers were counted and analyzed statistically by using the Wilcoxon paired sample test.

Chemicals (*Z*)-Pentacos-12-ene was synthesized by alkylation of 1-tridecyne with dodecyl triflate, modifying a procedure of Matsuda et al. (2002). After purification by column chromatography, unreacted 1-tridecyne was removed by washing with $AgNO_3$ solution, yielding pure 12-pentacosyne. Hydrogenation with Lindlar's catalyst and H_2 in pentane provided pure (*Z*)-pentacos-12-ene in 18% overall yield, containing less than 2% of the (*E*)-isomer.

Results and Discussion

Chloroform extracts of first-instar larvae showed strong oviposition deterrent activity in bioassays (Table 1). Analysis of the extract by GC/MS (Fig. 1) showed that the major compounds in the chromatogram, constituting up to 95% of the extract, were the alkaloids precocinelline and hippocasine, accompanied by mostly unbranched C_{23}–C_{35} hydrocarbons. However, NMR analysis revealed that the sample actually contained almost pure coccinelline (LeBrun et al. 1999), but this alkaloid proved to be inactive in our bioassay. The free amine alkaloids observed were formed in the injection port of the gas chromatograph from the alkaloid-*N*-oxide coccinelline. It might be possible that compounds not detectable by GC/MS may play a role as ODP. Therefore, we decided to fractionate the extract by chromatography on Sephadex LH-20. Six fractions were obtained, of which one showed a pronounced oviposition-deterrent activity. The investigation of this fraction by

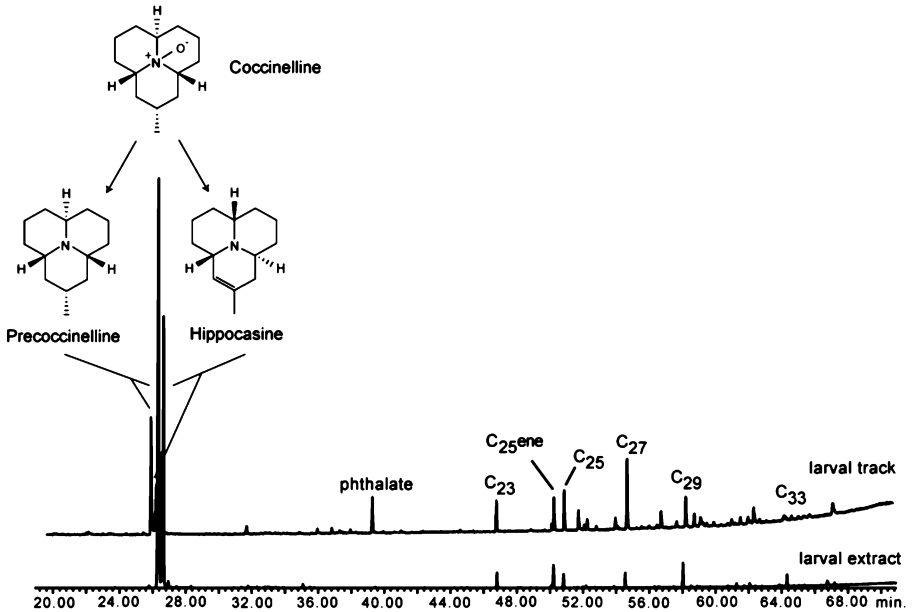


Fig. 1 Gas chromatogram of extracts of *Cheilomenes sexmaculata* first instars and a first-instar larval track

nanospray ESI-MS should reveal the presence of nonvolatile and/or polar compounds not analyzable by GC/MS, but no such compounds were found. Instead, we observed by GC/MS that this fraction contained a complex mixture of cuticular hydrocarbons. Major components in the fraction were pentacosane, heptacosane, and nonacosane, which were bioassayed individually and as a mixture in natural proportions but which were not active. An additional compound present in this fraction was a pentacosene, the only alkene occurring in the mixture in significant amounts. The position of the double bond was determined by using DMDS derivatization (Schulz et al. 2000). The formation of the DMDS adduct showed that the double bond was located at C-12, as evidenced by the strong α-cleavage ions at *m/z* 215 $(C_{12}H_{24}SCH_3)^+$ and *m/z* 229 $(C_{13}H_{26}SCH_3)^+$. Therefore, (*Z*)-pentacos-12-ene was synthesized by alkylation of 1-tridecyne with dodecyl triflate (Matsuda et al. 2002), followed by Lindlar-catalyzed hydrogenation. Gas chromatographic comparison showed that the natural compound did indeed have the (*Z*)-configuration because the two geometric isomers had different retention times on the BPX-5 phase used. In addition, (*Z*)-pentacos-12-ene was also found in extracts of tracks of first-instar larvae, proving the actual presence on the substrate (Fig. 1). This pentacosene showed considerable oviposition-deterrent activity in our bioassay. At high concentrations (1.25 μg/cm^2), the activity was strong, equivalent to the chloroform extract of 20 larvae per substrate. With lower concentrations (0.125 and 0.0125 μg/cm^2), the activity decreased as expected (Table 1). Nevertheless, because the activity of the extract was higher than that of the pentacosene, it is likely that other extract constituents are also active or might act synergistically with (*Z*)-pentacos-12-ene.

Our results prove that (*Z*)-pentacos-12-ene, most likely present on the cuticle and also found in tracks of the larvae, is an ODP of *C. sexmaculata*, the first such pheromone identified from coccinellids.

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Keywords Hessian fly · Mayetiola destructor · Gall midge · Host plant resistance · Wheat

Introduction

Understanding of plant defense via major resistance (*R*) genes and parasite adaptation via avirulence (*Avr*) genes has advanced considerably in the past decade. Numerous *R* genes from host plants and *Avr* genes from parasites have been cloned and characterized (Martin et al. 2003). In some cases, plant defense responses are triggered when an *R* protein recognizes a specific *Avr* elicitor (Dodds et al. 2006) in what is historically referred to as a gene-for-gene interaction (Flor 1955; Cook 1998). Plant defense can also be triggered by the action of general elicitors produced by the parasite or by the damaged plant (Nimchuk et al. 2003; Garcia-Brugger et al. 2006; Schmelz et al. 2006). Current understanding of *R*/*Avr* interactions is based almost entirely on studies of plant–pathogen systems.

In contrast, progress in plant–insect systems has been relatively slow. Only one insect *R* gene, *Mi-1.2*, has been cloned (Dogimont et al. 2003; Kaloshian and Walling 2005). The *Mi-1.2* gene, initially cloned because of its resistance to root-knot nematodes (Milligan et al. 1998), was found to confer resistance to both potato aphids and whiteflies (Rossi et al. 1998; Goggin et al. 2001; Nombela et al. 2003). Another putative insect *R* gene, the melon *Vat* gene for virus aphid (*Aphis gossypii*) transmission resistance, encodes a cytoplasmic protein with a nucleotide binding site and leucine-rich repeat (NBS-LRR) structure, but its effectiveness against insects has not yet been proven (Dogimont et al. 2003). Other genomic and molecular studies of plant responses to insect attack have revealed additional features, some of which are similar to those seen in plant–pathogen interactions (reviewed by Kalashian 2004; Kaloshian and Walling 2005; Thompson and Goggin 2006).

Among plant–insect systems, the wheat–Hessian fly (HF) [*Mayetiola destructor* (Say)] interaction is relatively well-characterized (reviewed in Harris et al. 2003). There are 32 *R* genes (designated *H1-H32*) that were originally found in wheat or its relatives. Both *R* genes and *Avr* genes confer dramatic differences in plant and parasite phenotypes. When attacked by virulent larvae, wheat seedlings without an effective *R* gene stop growing and often die without producing seed (Byers and Gallun 1972; Anderson and Harris 2006). It is likely that plant growth is suppressed because virulent larvae induce a nutritive tissue (i.e., a sink for photoassimilates) at the attack site (Harris et al. 2006). In contrast, *R* gene plants attacked by avirulent larvae can recover from an attack (Anderson and Harris 2006). Avirulent larvae invariably die 3 to 5 days after initiating attack of *R* gene plants. The wheat–HF interaction shows common features of the gene-for-gene model (Keen 1990; Harris et al. 2003). Thus, studies that use the traditional tools of Mendelian genetics have shown that each HF *Avr* gene corresponds with a plant *R* gene (Hatchett and Gallun 1970; Lobo et al. 2006). Analyses indicate that alleles at these *Avr* loci determine whether the insects will succeed in manipulating cell development and plant growth (the compatible interaction) or die without harming the plant (the incompatible interaction).

Molecular analyses of compatible and incompatible wheat–HF interactions are now proceeding. Several genes in wheat that encode lectin-like proteins including *Hfr-1* (Williams et al. 2002), *Hfr-2* (Puthoff et al. 2005), *Hfr-3* (Giovanini et al. 2007), and *Wci-1* (Subramanyam et al. 2006) are regulated differentially. *Hfr-1* and *Hfr-3* are up-regulated dramatically during incompatible interactions but only slightly during compatible interactions. *Hfr-2*, on the other hand, is only up-regulated during compatible interactions. *Hfr-1* and *Wci-1* are also up-regulated in response to other biotic and abiotic stresses (Subramanyam et al. 2006). In addition to these lectin-like genes, several other genes are

expressed differentially in wheat nearly isogenic lines with and without the *H21* gene (Jang et al. 2003). Given the dramatic physiological and phenotypic differences exhibited during compatible and incompatible wheat–HF interactions, it seems likely that these plant genes are not the only ones that respond to HF attack. In this paper, we report the global pattern of transcription during compatible and incompatible interactions, as revealed using the Affymetrix GeneChip® Wheat Genome Arrays.

Methods and Materials

Plant and Insect Materials Experimental design is shown in Table 1. Wheat near-isogenic lines "Iris" and "Molly" were provided by Dr. H. Ohm from Purdue University. Iris contains *R* gene *H9*, whereas Molly contains *R* gene *H13*. Both Iris and Molly were obtained via backcrossing for seven generations to the susceptible recipient "Newton" (Patterson et al. 1994). Because *H9* and *H13* are located on two different chromosomes that originated from different genomes (Liu et al. 2005a, b) but exhibit essentially the same phenotypic symptoms during compatible and incompatible interactions (Anderson and Harris 2006), their commonly up-regulated or down-regulated genes are likely to play a role in host-plant resistance or susceptibility.

The HF genotypes that include biotypes L, v*H9*, and v*H13* were maintained on Karl 92 (susceptible to all biotypes), Iris (*H9*), and Molly (*H13*), respectively, in the greenhouse. Biotype L is avirulent to both *H9* and *H13*. Biotype v*H9* is virulent to *H9* but avirulent to *H13*. Biotype v*H13* is virulent to *H13* but avirulent to *H9*. All three biotypes are virulent to Newton, the susceptible recipient parent.

Experimental Treatments and RNA Extraction Wheat seedlings were grown and infested with different biotypes in a growth chamber set at 20±1°C in daytime and 18±1°C at night with a 14:10 h (L/D) photoperiod. Seedlings were infested at two-leaf stage with eggs from 10 mated females per 20 plants. Larval hatching time was monitored with additional infested plants under the same growth chamber. Tissue samples from 20 plants in each replicate were collected and pooled 3 days after larval hatching. Only those plants with 10 to 15 larvae per plant were chosen for tissue collection. The time chosen for sample

Table 1 Experimental design

Wheat Lines	HF Biotypes	Phenotypes
Iris (*H9*)	L	Incompatible
	vH9	Compatible
	Uninfected	Control
Molly (*H13*)	L	Incompatible
	vH13	Compatible
	Uninfected	Control
Newton (S)	L	Compatible
	Uninfected	Control

Iris (*H9*) and Molly (*H13*) were infested with two biotypes: *L* (avirulent) and *vH9* or *vH13* (virulent). Newton was infested with biotype *L* (virulent). Three independent biological replicates were included for each treatment. The data obtained from each treatment were compared with the corresponding uninfested control within each wheat line under identical conditions.

collection was based on the biology that the third day is critical to determine whether a larva can live or will die on a specific plant (Byers and Gallun 1972). Only the tissue at the feeding site (about 1.5 to 2 cm) of the second leaf sheath was collected for RNA extraction. The *H9* seedlings infested with v*H9* or L were defined as *H9*-compatible (*H9*-C) or *H9*-incompatible (*H9*-I), respectively; the *H13* seedlings infested with biotypes v*H13* or L were defined as *H13*-compatible (*H13*-C) or *H13*-incompatible (*H13*-I), respectively. Seedlings of each wheat genotype under the same conditions but without infestation were used as uninfected control. Each experiment contained three independent sets of seedlings that were treated in the same way at different times in different chambers as biological replicates. A portion of the seedlings in each experiment was kept for phenotypic confirmation later.

Total RNA was extracted from wheat tissues by using TRI reagent™ according to the procedure provided by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH, USA). The RNA samples were further purified through an RNease kit according to the manufacturer's instruction (Qiagen Inc., Valencia, CA, USA). The RNA concentrations were measured with a NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was quantified using Agilent 2100 Bioanalyzer (Agilent, Foster City, CA, USA).

Microarray Hybridization and Data Analysis GeneChip® wheat genome arrays containing 61,127 probe sets were purchased from Affymetrix (Santa Clara, CA, USA). Labeling and hybridization on the wheat microarrays were performed according to the standard protocol provided by Affymetrix. (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). After hybridization and washing, the arrays were scanned with an Affymetrix GeneChip Scanner 3000. Hybridization quality was verified by scaling factor, overall hybridization rate, and signal strength of several bacterial spike controls. The spike controls were hybridized with labeled targets in different concentrations resulting in particular ratios between different spikes.

Image acquisition, image setting, and raw data generation were done with Affymetrix GeneChip® Operating Software (GCOS). After alignment of the image settings for each chip, raw data were extracted and marked as "present" (well above the background), "marginal," or "absent" (similar to or below the background) under default settings. The overall target signal intensity of each chip was scaled to 500 and then loaded into GeneSpring GX 7.3.1 software (Agilent Technologies, Palo Alto, CA, USA) for further analysis. The data were prenormalized to the 50th percentile per chip and then normalized based on per-gene normalization with the median method, from which the signal of each gene in a particular sample was divided by the median signal of the same gene in all samples. In the subsequent paired comparison between the experimental treatment and the corresponding uninfected control, only those transcripts (genes) that were declared "present" for at least three out of six chips within a given triplicate comparison were retained for further analysis. The genes that showed significant changes in their expression in treated and control plants were selected by applying a *t* test (one-way analysis of variance with "Benjamini and Hochberg False Discovery Rate" for multiple comparisons, $P=0.05$). The fold-change values were calculated between the treatment and control samples on the basis of the normalized average measurement of the signal intensity. A cutoff value of twofold change, which is commonly used for microarray analysis (Eisen et al. 1998), was adopted to discriminate expression of genes that were differentially altered in response to HF infestation. Annotation of the genes represented on the microarray to genomic open reading frames was done with programs of HarvEST: Wheat v1.13 (http://www.harvest.ucr.edu/), Affymetrix NetAffx, and GeneSpring. The functions of the genes listed in Tables 3

to 8 were further manually updated by searching the GenBank with BLASTX. Cluster analysis was carried out according to Eisen et al. (1998) using the current version of Cluster/TreeView programs at http://www.rana.stanford.edu/clustering.

Northern Blot Validation and Correlation Analysis Five micrograms of total RNA of each sample were separated on 1.5% agarose gels that contained formaldehyde and were blotted onto GeneScreen membrane (Perkin Elmer, Boston, MA, USA). Membranes were baked at 80°C for 2 h to fix the RNA to the membrane, and then were hybridized with complementary DNA (cDNA) probes labeled with ^{32}P-dCTP using the random labeling kit from Stratagene (La Jolla, CA, USA), separately. Specific cDNAs used for generating probes were isolated through PCR amplification of reverse-transcripts derived from a messenger RNA (mRNA) sample purified from wheat sheath by using a pair of primers synthesized according to the corresponding expressed sequence tag sequences. Hybridization was carried out overnight at 42°C in a plastic bag containing 15 ml hybridization solution (10% dextran sulfate/1% sodium dodecyl sulfate (SDS)/1 M NaCl, pH 8.0) as described by Chen et al. (2004). After hybridization, the membranes were washed twice with 2× saline-sodium citrate (SSC) at room temperature for 30 min, twice with 2× SSC plus 1% SDS at 65°C for 30 min, and twice with 0.1× SSC plus 0.1% SDS at room temperature for 30 min. The membranes were then exposed to Kodak SR-5 X-ray film for 1 to 3 days, depending on signal intensity. Both autoradiographs and the 18S ribosomal RNA (rRNA) band on the negatives of the photographs of agarose gels used for Northern blot analysis were quantified by scanning laser densitometry with an Ultroscan XL laser densitometer and the GSXL2 software package (Pharmacia). Densitometry signals from exposed X-ray films were the average of the three readings.

Results

Overview Among a total of 61,127 probe sets contained in the microarrays, 25,246 (41%) produced detectable hybridization signals under our experimental conditions. To identify genes expressed differentially under various conditions, we use the twofold cut-off criterion, which is used in most studies. Among the 25,246 probe sets that produced detectable signals, 4,112 (16%) and 3,516 (14%) detected at least twofold changes (either up or down) between non-attacked and attacked *H9* plants and between non-attacked and attacked *H13* plants, respectively. The distribution of these differentially expressed genes during compatible or incompatible interactions is shown in Table 2. In general, the number of genes that were up-regulated was about three times greater than the number of genes that were down-regulated during both compatible and incompatible interactions. The number of genes that were either up- or down-regulated during compatible interactions was ~1.5 times greater than during incompatible interactions.

Comparative analysis of the differentially expressed genes identified 1,227 genes that were commonly affected in both *H9* and *H13* plants: 629 genes with altered expression during compatible interactions, 584 genes with altered expression during incompatible interactions, and 14 genes with altered expression during both compatible and incompatible interactions. Joint analyses of these commonly up- or down-regulated genes revealed six expression clusters (Fig. 1). Cluster I contained genes down-regulated during compatible interactions (Dc) and cluster II contained genes up-regulated during incompatible interactions (Ui). The altered expression of the genes in clusters I and II resulted in higher

Table 2 Distribution of genes expressed differentially in response to HF infestations

Fold Change	*H9-C*	*H13-C*	*H9-I*	*H13-I*
Up-regulated				
2~4	1,343	1,423	811	903
4~8	542	336	388	251
>8	404	136	273	147
Total	2,289	1,895	1,472	1,301
Down-regulated				
<1/8	56	28	10	51
1/8~1/4	139	74	36	59
1/4~1/2	557	394	322	251
Total	752	496	368	361

H9-C, *H13*-C, *H9*-I, and *H13*-I represent *H9*-compatible, *H13*-compatible, *H9*-incompatible, and *H13*-incompatible, respectively.

levels of expression during incompatible interactions than those during compatible interactions. The genes with known functions in clusters I and II mainly belong to six functional categories: antibiotic defense-related including protease inhibitors and defensins; phenylpropanoid metabolism-related including various *O*-methyltransferases, isoflavone

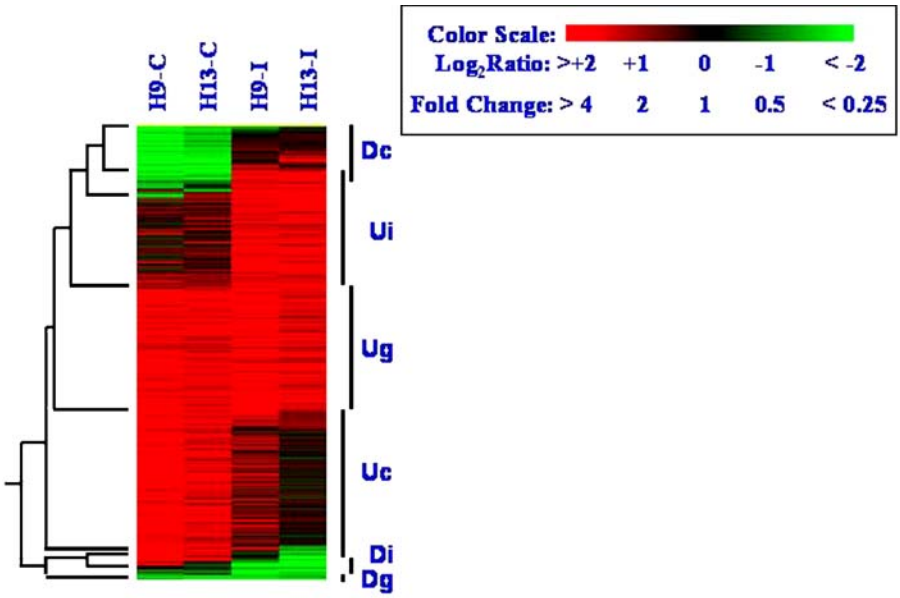


Fig. 1 Cluster display of genes expressed differentially during compatible and incompatible interactions. *H9-C, H13-C, H9-I*, and *H13-I* represent *H9*-compatible, *H13*-compatible, *H9*-incompatible, and *H13*-incompatible interactions, respectively. Six clusters were defined as *Dc* down-regulated during compatible interactions, *Ui* up-regulated during incompatible interactions, *Ug* up-regulated generally during both compatible and incompatible interactions, *Uc* up-regulated during compatible interactions, *Di* down-regulated during incompatible interactions, *Dg* down-regulated generally during both compatible and incompatible interactions. The cut-off for up- or down-regulation is ±2-fold

reductases, chalcone synthases, betanidin-*O*-glucosyltransferases, and flavanone 3-hydroxylases; lipid metabolism-related including lipid transfer proteins, lipases, and other enzymes; oxidation-related including glutathione transferases, peroxidases, and cytochrome P450; cell wall metabolism-related including cellulose synthases, xyloglucan endotransglycosylases (XET), glucanases, expansins, pectinesterases, and chitinases; and genes that encode various histones. Cluster III contained genes up-regulated during both compatible and incompatible interactions (Ug). Genes in this cluster are involved in various cellular processes including metabolism, transport, and regulation of gene expression and/or signal transduction. Cluster IV contained genes up-regulated during compatible interactions (Uc), and cluster V contained genes down-regulated during incompatible interactions (Di). The altered expression of the genes in clusters IV and V resulted in higher expression levels during compatible interaction than those during incompatible interactions. The genes with known functions in clusters IV and V mainly belong to three functional categories: genes involved in stress response, including various heat-shock proteins; genes involved in nutrient metabolism and transport; and genes involved in regulation and/or signal transduction. Cluster VI contained genes that were down-regulated during both compatible and incompatible interactions (Dg). There are only a few genes in this cluster.

Data Validation The microarray results were validated via Northern blots with two types of samples: the same RNA samples that were used for microarray analysis and newly prepared RNA samples from independent infestations. Fifty-two comparable pairs of microarray and Northern blot data sets were obtained from the same RNA preparations. The 52 data sets produced a 0.98 correlation ($P<0.001$) between microarray results and Northern blot data (Fig. 2). The high correlation suggests that the microarray data were reliable. Northern blots were also conducted with RNA samples prepared from independent infestations with multiple time points (Fig. 3). Again, the Northern blot results are generally in agreement with microarray data.

Analysis of Genes Responded Differentially to HF Attack We analyzed the genes that appeared to be major targets for differential regulation during compatible and incompatible interactions. These genes could be sorted into seven groups: genes involved in antibiotic defense, genes involved in the phenylpropanoid pathway, genes involved in cell wall metabolism, genes involved in lipid metabolism, genes involved in nutrient metabolism and transport, genes that encode histones and cellular structural proteins, and genes that encode heat-shock proteins and ubiquitins.

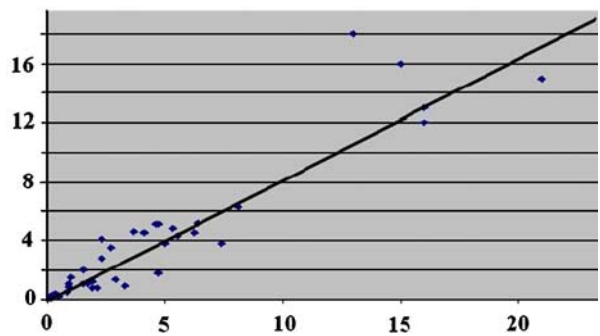
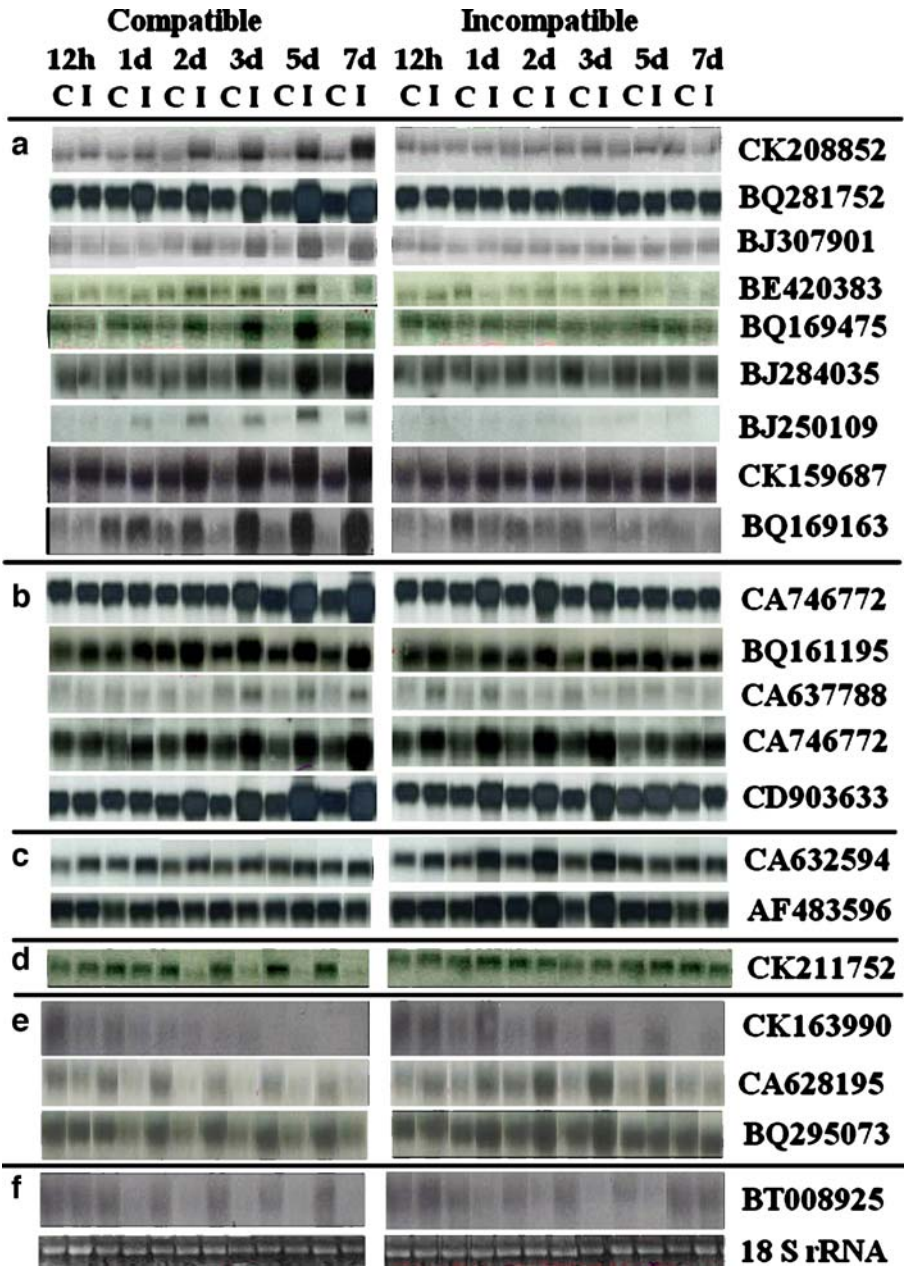


Fig. 2 Correlation analysis of microarray and Northern blot data sets. The same RNA samples were used in both microarray and Northern blot experiments



Genes Involved in Antibiotic Defense Table 3 lists genes that either encode proteins that are toxic to insects directly or encode an enzyme that participates in producing toxic molecules. The genes can be classified into three groups: genes that encode protease inhibitors (some of them also sharing sequence similarity with defensins or thionin), the genes that encode jacalin-like lectins or horcolins that have a jacalin-like lectin domain, and a gene that

◀ **Fig. 3** Northern blot analyses with RNA samples obtained from independent infestations. RNA samples were prepared from susceptible (compatible) and resistant (incompatible) plants at 12 h (*12h*), 1 day (*1d*), 2 days (*2d*), 3 days (*3d*), 5 days (*5d*), and 7 days (7d) after initial HF attack, respectively. *C* represents control plants whereas *I* presents infested plants. The accession numbers of cDNA probes were given on the *right side* of the figure. The image of 18 S rRNA was given to demonstrate relatively equal loadings. The blots were sorted into different groups: **a** genes up-regulated during compatible interactions, **b** genes up-regulated during both compatible and incompatible interactions, **c** genes up-regulated during incompatible interactions, **d** a gene down-regulated during compatible interactions, **e** genes down-regulated during compatible interactions but up-regulated during incompatible interactions, and **f** a gene down-regulated during both compatible and incompatible interactions. **b** Correlation analysis of Northern blot results and microarray data with 20 randomly selected cDNA probes

encodes a mandelonitrile lyase (an enzyme participating in hydrogen cyanide production). All these genes were expressed at least twofold higher levels during incompatible interactions than during compatible interactions.

Genes Involved in the Phenylpropanoid Pathway The first committed step in the biosynthesis of phenylpropanoids and other phenolic compounds is the conversion of L-phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia-lyase (PAL; Rasmussen and Dixon 1999). Seven out of eight related cDNAs (with 65 to 96% sequence identity) that encode PALs were strongly up-regulated (4- to 46-fold) during both compatible and incompatible interactions (Table 4). The eighth one (BJ318940) was also up-regulated slightly during both types of interactions. The up-regulation patterns among the genes were different between compatible and incompatible interactions: Three of the cDNA probes detected lower levels of up-regulation during incompatible interactions (an I/C ratio 0.5 to 0.72), whereas the rest of the cDNA probes detected higher levels of up-regulation during incompatible interactions (an I/C ratio 1.35 to 2.7).

Table 3 Expression levels of genes that encode putative defense molecules

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
BT008925	2.558	0.127	0.151	0.277	0.434	Defensin/thionins/protease inhibitor
BJ314514	3.146	0.621	1.16	2.641	2.961	Bowman–Birk type trypsin inhibitor
CK200200	6.544	0.276	0.263	1.644	1.883	Protease inhibitor
BJ316836	7.398	0.0457	0.103	0.397	0.703	Protease inhibitor II/defensin
CA652325	11.902	0.987	1.259	18.04	8.69	Defensin/thionin-like protein
CK152019	12.611	0.186	0.145	2.08	2.094	Protease inhibitor
BJ314399	13.129	0.0332	0.141	0.841	1.446	Defensin/gamma-thionins /protease inhibitor
BJ320233	4.181	1.32	0.701	4.038	4.411	Jacalin lectin family protein
BU672305	4.237	1	0.77	3.115	4.383	Jacalin lectin family protein
AF483596	4.664	3.322	4.043	15.46	18.89	Jacalin-like lectin, HF response gene-1 protein
CD887397	6.747	0.855	3.258	14.37	13.38	Horcolin, Jacalin-like lectin domain
CA676018	10.672	0.176	0.22	2.361	1.865	Horcolin, Jacalin-like lectin domain
CK152094	6.078	0.0631	0.277	0.564	1.503	Mandelonitrile lyase (hydrogen cyanide production)

Gene IDs are the accession numbers from GenBank. I/C ratio represents the ratio of average fold change between incompatible and compatible interactions. *H9-C*, *H13-C*, *H9-I*, and *H13-I* represent fold changes in *H9* compatible, *H13* compatible, *H9* incompatible, and *H13* incompatible interactions, respectively, in comparison with the corresponding controls.

Table 4 Expression levels of genes involved in phenylpropanoid metabolism

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
CK197611	0.503	26.55	11.59	14.43	4.726	Phenylalanine ammonia-lyase
CK163994	0.556	32.01	12.93	18	6.981	Phenylalanine ammonia-lyase
BQ161844	0.72	6.485	5.178	4.835	3.554	Phenylalanine ammonia-lyase
CK210556	1.345	4.942	3.844	5.992	6.026	Phenylalanine ammonia-lyase
CK198174	1.42	32.16	11.99	46.8	15.85	Phenylalanine ammonia-lyase
BJ318940	1.505	1.495	1.578	2.189	2.435	Phenylalanine ammonia-lyase
BJ319555	1.128	7.873	8.803	9.628	9.171	Phenylalanine ammonia-lyase
BF199967	1.525	6.102	5.186	8.502	8.704	Phenylalanine ammonia-lyase
BJ281221	2.693	4.751	3.903	12.22	11.08	Phenylalanine ammonia-lyase
CD373447	5.473	0.348	0.257	1.725	1.586	Caffeic acid *O*-methyltransferase
CK214164	18.943	0.127	0.36	3.476	5.749	Caffeic acid *O*-methyltransferase
BJ283780	1.216	8.449	8.935	10.17	10.97	*O*-methyltransferase-like
CK203071	5.336	0.186	0.151	0.778	1.02	*O*-methyltransferase
CA626582	6.32	2.778	2.308	14.05	18.09	*O*-methyltransferase
CA667822	6.508	0.333	0.509	3.174	2.305	*O*-methyltransferase
U76384	9.309	0.132	0.121	1.144	1.211	*O*-methyltransferase
CK163990	12.631	0.395	0.298	5.313	3.44	*O*-methyltransferase
BJ256875	33.658	0.363	0.356	10.01	14.19	*O*-methyltransferase
CA635144	42.726	0.0236	0.0718	0.957	3.119	*O*-methyltransferase
CK200910	65.981	0.05	0.055	3.994	2.934	*O*-methyltransferase
BJ256875	85.017	0.088	0.209	13.69	11.56	*O*-methyltransferase
BQ168386	220.192	0.0102	0.0212	1.419	5.495	*O*-methyltransferase
CK154453	155.092	0.00758	0.0262	1.35	3.889	Isoflavone *O*-methyltransferase
CK196344	4.093	0.353	0.198	1.021	1.234	Flavonoid 7-*O*-methyltransferase
CB307103	372.076	0.00479	0.00581	1.454	2.49	Flavonoid 7-*O*-methyltransferase
BQ578840	2.365	0.859	1.98	2.86	3.854	Betanidin-5-*O*-glucosyltransferase
CA637965	2.466	1.009	2.576	3.652	5.188	Betanidin-5-*O*-glucosyltransferase
BJ265841	3.613	0.228	0.453	0.935	1.525	Isoflavone reductase
CA674957	7.006	0.789	1.143	5.989	7.546	Flavanone 3-hydroxylase
CK208240	7.673	0.376	0.164	2.824	1.319	Flavonoid 3-hydroxylase
CK155175	4.01	0.268	0.442	0.867	1.98	Chalcone synthase
CA718360	33.767	0.118	0.0591	4.705	1.275	Chalcone synthase

Table legends are as defined in Table 3.

A group of enzymes called *O*-methyltransferases (OMT) participate in various steps in the phenylpropanoid metabolism pathway, including the step that converts caffeic acid into ferulic acid (Dixon et al. 2002). Ferulic acid is a substrate for the biosynthesis of lignin, an important component of cell-wall polymers. In contrast to PAL-encoding genes that were up-regulated during both compatible and incompatible interactions, all OMT cDNAs except two (BJ283780 and CA626582) detected strong down-regulation of the corresponding genes during compatible interactions. These OMT genes were either not affected or up-regulated during incompatible interactions. A combination of down-regulation during compatible interactions and up-regulation during incompatible interactions resulted in significant higher expression of these genes during incompatible interactions (I/C ratio 4 to 372) than that during compatible interactions. The two exceptions also exhibited different expression patterns: CA626582 exhibited a slight up-regulation during compatible interactions and a strong up-regulation during incompatible interaction, whereas

BJ283780 exhibited strong up-regulation during both compatible and incompatible interactions. Several other genes that encode enzymes involved in the metabolism of chalcone, flavonoids, isoflavonoids, and other secondary metabolites exhibited expression similar to those of patterns as the OMT genes during compatible and incompatible interactions.

Genes Involved in Cell-Wall Metabolism In general, enzymes involved in cell-wall loosening or in basic component synthesis exhibited higher expression levels during incompatible interactions than those during compatible interactions (IC ratio 1.5 to 151, Table 5). Enzymes involved in cell-wall loosening during growth include XET, β-expansin, pectinesterase, polygalacturonase, and endo-1,4-β glucanases (Cosgrove 2003). Enzymes involved in basic cell-wall component synthesis include cellulose synthase, eceriferum (CER1; involved in the conversion of long-chain aldehydes to alkanes, a key step in wax biosynthesis, Aarts et al. 1995), and dirigent, which participates in lignan and lignin biosynthesis (Davin and Lewis 2005).

On the other hand, several genes exhibited lower expression during incompatible interaction than that during compatible interactions (I/C ratio, 0.12 to 0.72, Table 4). These genes encode, respectively, pectin-glucuronyltransferase, which participates in synthesis of pectin (Iwai et al. 2002); pectinacetylesterase, which catalyzes the deacetylation of pectins (Vercauteren et al. 2002); xyloglucan fucosyltransferases involved in hemicellulose xyloglucan branching (Perrin et al. 1999); and arabinofuranohydrolase, which hydrolyzes arabinoxylan (Ferre et al. 2000).

Table 5 Expression levels of genes involved in cell-wall metabolism

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
Genes with higher I/C ratio						
CK212850	1.506	3.591	2.938	5.585	4.245	Xyloglucan endotransglycosylase
CN011869	3.805	0.68	1.467	3.944	4.224	Xyloglucan endotransglycosylase
BF199968	4.382	0.26	0.348	1.268	1.396	Xyloglucan endotransglycosylase
CA606310	6.025	0.66	1.149	3.367	7.531	Xyloglucan endotransglycosylase
BJ249762	7.766	0.429	0.395	3.381	3.018	β-Expansin TaEXPB4
CK167447	12.624	0.556	1.002	6.037	13.63	β-Expansin TaEXPB4
CA632215	2.018	0.818	1.849	2.341	3.041	Pectinesterase
CA497753	2.831	1.28	1.511	4.87	3.031	Pectinesterase
BQ170792	11.145	0.375	0.225	4.182	2.505	Polygalacturonase A
BJ311097	2.6	0.442	0.495	1.285	1.151	Endo-1,4-β glucanase
BE446813	1.928	0.981	1.263	2.234	2.091	Glucanase
BJ264002	5.848	0.763	0.697	5.656	2.881	Cellulose synthase
CK165915	2.349	0.294	0.262	0.643	0.663	Cellulose synthase
CK208158	27.808	0.289	0.507	12.36	9.775	CER1-like protein
CD452431	11.19	0.338	0.295	2.87	4.213	Dirigent-like protein
BJ227727	151.523	0.029	0.128	20.82	2.969	Dirigent-like protein
Genes with lower I/C ratio						
CA636147	0.345	6.555	3.248	2.249	1.132	Pectin-glucuronyltransferase
BJ231486	0.6	2.958	2.685	1.304	2.081	Pectinacetylesterase
CK199494	0.472	6.079	4.623	2.691	2.355	Xyloglucan fucosyltransferase
CA641288	0.457	5.624	5.116	2.846	2.053	Xyloglucan fucosyltransferase
BJ275014	0.12	10	4.437	1.182	0.557	Arabinoxylan arabinofuranohydrolase

Table legends are as defined in Table 3.

In addition to the genes listed in Table 5, several other genes are involved in cell-wall metabolism including chitinases, β-1,3-glucanases, glucan 1,3-β-glucosidases, and a xylanase inhibitor. These enzymes and enzyme inhibitors are typically associated with fungal cell-wall metabolism and may be used as defensive weapons by the plant against secondary fungal invasion.

Genes Involved in Lipid Metabolism Genes involved in lipid metabolism can be classified into five groups based on their encoding proteins: lipid transfer proteins; lipases; enzymes involved in the biosynthesis of fatty acids, wax, or phytohormones; enzymes involved in fatty acid degradation or energy metabolism; and enzymes involved in phospholipid metabolism (Table 6).

All lipid transfer protein genes exhibited higher expression during incompatible interactions than during compatible interaction (I/C ratio, 1.71 to 24.2). Similarly, all lipase genes except two (BG605132 and BJ272793) exhibited higher expression levels during incompatible interactions (I/C ratio, 1.77 to 8.92). The two exceptions, BG605132 and BJ272793, which encode a lipase-like protein and a triacylglerol lipase, respectively, exhibited lower levels of expression during incompatible interaction (I/C ratio, 0.315 and 0.806, Table 6). In addition, all the genes that encode enzymes involved in the biosynthesis of fatty acids, wax, or other substances also exhibited higher expression levels during incompatible interactions (I/C ratio, 1.46 to 24.166). In contrast, all genes with one exception involved in fatty acid degradation and phospholipid metabolism exhibited lower expression during incompatible interactions than during compatible interaction (I/C ratio, 0.105 to 0.887). The exception, CK213159, which encodes a lipoxygenase, was strongly up-regulated during both compatible and incompatible interactions with an I/C ratio of 1.2.

Genes Involved in Nutrient Metabolism and Transport Genes involved in nutrient metabolism and transport were expressed at much higher levels during compatible interactions than during incompatible interactions (I/C ratio from 0.04 to 0.75, Table 7). Most of the genes that encode metabolic enzymes and nutrient transporter were strongly up-regulated during compatible interactions but were not affected or even down-regulated during incompatible interactions.

Genes that Encode Histones and Cellular Structural Proteins Sixteen different histone genes, together with a histone acetyltransferase B gene, were consistently down-regulated during compatible interactions (Table 8). These histone genes either were not affected or were slightly up-regulated during incompatible interactions. The combination of down-regulation during compatible interactions and up-regulation during incompatible interactions resulted in significantly higher expression of these genes during incompatible interactions (I/C ratio, 2.28 and 7.78).

Although the genes that encode histones, the chromatin structural proteins, were down-regulated during compatible interactions, the genes that encode cellular structural proteins including actins and α-tubulin, were highly (2- to 22-fold) up-regulated during compatible interactions. The actin and tubulin encoding genes either were not affected or were only slightly up-regulated during incompatible interactions.

Genes that Encode Heat-Shock Proteins and Ubiquitins There were 12 differentially expressed genes that encode various heat-shock proteins (*HSP*; Table 8). During compatible interactions, all these *HSP* genes were expressed at higher levels than those during incompatible interactions (I/C ratio, 0.004 to 0.494). With one exception (AJ603283), all

Table 6 Expression levels of genes involved in lipid metabolism

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
Genes encoding lipid transfer proteins						
CA630456	1.714	2.657	1.678	5.09	2.34	Lipid transfer protein
CN012788	1.949	1.578	1.258	3.072	2.454	Type 1 non-specific lipid transfer protein
BG907205	2.544	1.328	0.955	2.974	2.832	Lipid transfer protein, putative
CD896324	3.373	1.775	0.579	5.292	2.647	Lipid transfer protein, putative
CA631890	3.62	0.101	0.22	0.342	0.82	Lipid transfer protein-like protein
CA674677	3.849	0.606	2.376	5.091	6.386	Lipid transfer protein (LTP) family protein
CK164223	3.969	0.744	0.921	3.144	3.464	Non-specific lipid transfer protein 1
CA632594	5.1	0.935	1.115	4.626	5.828	Type 1 non-specific lipid transfer protein
CA635994	6.611	1.337	1.088	7.975	8.055	Type 1 non-specific lipid transfer protein
AF302788	6.702	0.976	0.785	7.291	4.511	Type 1 non-specific lipid transfer protein
AF334185	14.352	1.254	1.166	16.58	18.15	Lipid transfer protein
BQ838076	17.831	0.0931	0.43	2.527	6.8	Lipid transfer protein, putative
Genes encoding lipases						
BG605132	0.315	4.761	3.765	1.527	1.157	Lipase-like
BJ272793	0.806	2.208	4.035	2.519	2.513	Triacylglycerol lipase
CD453855	1.77	1.448	1.998	2.801	3.295	Pepper esterase/lipase, putative
AJ614438	2.285	1.362	1.406	3.716	2.607	Lipase
BE493028	2.899	1.173	4.679	6.246	10.72	Triacylglycerol lipase
BJ227857	3.768	2.744	2.25	10.69	8.123	Lipase/acylhydrolase
BJ246034	3.982	0.659	1.993	3.545	7.013	GDSL-motif lipase
CA671780	4.195	0.398	0.277	0.998	1.833	Lipase class 3-like
CK209259	4.021	0.248	0.43	1.315	1.411	Family II lipase EXL4, putative
CK215006	6.022	0.456	0.524	2.689	3.212	Family II extracellular lipase
BQ171153	8.921	0.751	0.829	9.998	4.097	Family II extracellular lipase 1
Genes encoding enzymes involved in biosynthesis of fatty acids, wax, or others						
AJ614506	1.46	1.45	2.786	2.585	3.601	Allene oxide cyclase
BJ241555	1.524	1.954	3.562	3.367	5.04	Allene oxide cyclase
CA681727	1.546	1.415	3.44	3.063	4.444	Omega-3 fatty acid desaturase
CA616263	1.616	2.005	2.833	3.428	4.39	Omega-3 fatty acid desaturase
CD454643	2.316	1.712	1.756	4.668	3.362	Fatty acyl CoA reductase
CK216702	2.356	1.31	1.513	3.158	3.491	β-Ketoacyl–CoA synthase
AJ459251	2.455	1.03	1.037	2.942	2.131	Fatty acyl CoA reductase
CA740940	3.098	0.922	1.451	3.924	3.427	Fatty acyl CoA reductase
BQ483035	5.276	0.159	0.266	1.005	1.237	Cyclopropane synthase
CA666657	13.36	1.331	2.556	18.82	33.11	Acetyl–CoA C-acyltransferase
CA630824	24.166	0.117	0.287	3.448	6.315	Acyl-activating enzyme 12, AMP-binding
Genes encoding enzymes involved in fatty acid degradation or energy metabolism						
CK205756	0.285	3.924	10.32	1.27	2.788	Carnitine/acylcarnitine translocase-like
BJ287245	0.439	2.744	4.121	1.365	1.643	Carnitine/acylcarnitine translocase-like
CD373536	0.351	1.011	0.548	0.28	0.267	Acyl–CoA oxidase (ACX2)
CK209068	0.388	3.729	2.737	1.447	1.061	Enoyl–CoA hydratase-like
CA624206	0.657	0.199	0.246	0.187	0.105	Lipoxygenase
CK213159	1.201	23.99	11.64	30.92	11.87	Lipoxygenase
AF542186	0.887	0.385	0.429	0.379	0.343	Cytochrome b5 reductase
Genes involved in phospholipid metabolism						
BQ169721	0.2	9.142	2.115	1.594	0.66	Choline kinase
CA681065	0.373	14.57	8.485	6.339	2.264	Putative phosphodiesterase
CA681065	0.417	22.41	4.693	9.472	1.806	Glycerophosphodiester phosphodiesterase
BQ171283	0.464	3.793	5.112	2.195	1.931	Phosphatidylglycerol specific

Table 6 (continued)

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
						phospholipase C
BJ231883	0.473	4.058	5.008	2.284	1.998	Bifunctional phosphopantetheine adenylyl transferase
BQ162554	0.597	3.139	2.297	1.664	1.577	Phosphatidylinositol 3,5-kinase-like

Table legends are as defined in Table 3.

these genes were up-regulated during compatible interactions, and some of them were dramatically up-regulated (up to 200-fold).

Another group of stress-related cDNAs, which encode ubiquitins or ubiquitin-pathway proteins, exhibited expression patterns similar to those of the heat-shock protein genes. These ubiquitin pathway genes exhibited higher expression levels during compatible interactions (I/C ratio, 0.048 to 0.861).

Table 7 Expression levels of genes involved in nutrient metabolism and transport

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
Nutrient metabolic enzymes						
BQ169475	0.152	20.86	12.1	2.909	2.097	Alpha-glucosidase
BQ170874	0.617	5.896	4.687	3.711	2.823	Neutral invertase
CD903633	0.279	16.02	6.355	4.695	1.54	Pyruvate dehydrogenase E1 alpha
BJ307901	0.356	2.708	6.268	1.458	1.737	Alcohol dehydrogenase
CK208852	0.562	3.981	2.452	1.834	1.782	Enolase
BJ313281	0.261	1.548	1.123	0.336	0.36	Electron transfer oxidoreductase
BE446498	0.105	32.78	1.123	0.336	0.36	Electron transfer oxidoreductase
BQ161195	0.279	8.103	16.7	4.047	1.129	Succinate:ubiquinone oxidoreductase
CA657311	0.363	4.401	6.065	2.087	1.865	Oxidoreductase, putative
CA653416	0.441	3.516	3.005	1.58	1.102	Flavin-binding monooxygenase
BJ250109	0.495	2.172	2.808	1.627	1.16	Aspartate aminotransferase
CN012484	0.751	7.493	3.203	0.959	1.698	Methionine synthase
Nutrient transporters						
CD931721	0.341	9.231	9.842	1.648	4.852	Sugar transporter
BJ320954	0.704	4.877	2.332	3.212	1.863	Sugar transporter
BJ275570	0.461	5.352	6.278	3.233	2.113	Sugar transporter
CD892342	0.347	12.86	6.388	4.382	2.112	Glucose translocator
BJ231104	0.491	4.323	2.782	1.953	1.584	Glucose-6-phosphate translocator
BQ171988	0.181	7.881	2.675	1.231	0.683	Myo-inositol transporter
BT009318	0.04	76.7	14.67	2.998	0.592	Ammonium transporter
CA601803	0.165	20.33	4.873	3.442	0.715	Ammonium transporter
BJ275567	0.096	6.992	5.419	0.75	0.436	Amino acid transporter
CA737017	0.236	153.2	5.741	35.82	1.663	Amino acid permease
CA638660	0.598	2.547	2.763	1.542	1.635	Proline transport protein 1
CK205233	0.0954	148.9	12.83	13.65	1.786	Proline transporter
BJ286275	0.192	163.7	15.36	32.12	1.874	Histidine transporter
BT009250	0.208	24.87	3.423	4.763	1.106	Histidine transporter

Table legends are as defined in Table 3.

Table 8 Expression levels of genes that encode structural proteins or stress proteins

Gene ID	I/C Ratio	H9-C	H13-C	H9-I	H13-I	Putative Identification
Genes encoding histones						
CK211752	3.28	0.218	0.436	1.047	1.098	Histone H3
BJ227379	3.366	0.289	0.43	1.135	1.285	Histone H4
BJ309223	4.028	0.26	0.321	1.379	0.961	Histone H4
BJ277068	4.059	0.253	0.442	1.339	1.482	Histone H4
BJ308966	4.153	0.29	0.41	1.48	1.427	Histone H4
BJ321665	4.259	0.255	0.384	1.121	1.6	Histone H4
BJ220049	4.371	0.281	0.394	1.429	1.521	Histone H4
CA728847	4.518	0.308	0.399	1.376	1.818	Histone H4
D38087	4.746	0.183	0.398	1.333	1.424	Histone H2A
BJ239910	4.886	0.283	0.431	1.356	2.132	Histone H4
BJ219603	4.923	0.275	0.42	1.549	1.872	Histone H4
BJ306762	5.359	0.204	0.377	1.211	1.902	Histone H2B-8
BJ240295	6.321	0.192	0.369	1.316	2.23	Histone H4
BJ228253	7.362	0.0739	0.359	0.967	2.22	Histone H2A.1
BF200454	7.78	0.164	0.307	1.266	2.398	Histone H4
CA652677	3.415	0.569	0.929	2.395	2.72	Histone acetyltransferase HAT B
Genes encoding actins and alpha-tubulin						
CK159687	0.115	21.87	5.654	2.058	1.093	Actin
CK160285	0.232	10.15	3.111	1.959	1.115	Actin
CK163937	0.306	4.377	5.329	1.982	0.988	Actin
CA616245	0.503	4.493	3.587	2.439	1.623	Actin
BJ287163	0.508	5.813	4.408	3.554	1.637	Putative actin
BJ276412	0.412	3.556	2.1	1.302	1.023	Alpha-tubulin
Genes encoding heat-shock proteins						
CD453475	0.004	199.3	30.27	0.792	0.0557	Putative heat-shock protein (HSP18.1-CI)
BJ296188	0.021	29.44	14.59	0.627	0.294	Heat-shock protein HSP70
AF104108	0.1	18.81	9.715	1.938	0.908	Heat-shock protein Hsp23.6
CA702016	0.11	6.682	4.78	0.828	0.424	Heat-shock protein 70
CA702016	0.145	6.503	4.133	1.041	0.494	Heat-shock protein 70
CA593468	0.187	9.164	9.545	2.158	1.326	Heat-shock protein 16.9
AJ603283	0.2	0.208	1.167	0.133	0.141	Small heat-shock protein
AJ614980	0.223	3.336	5.128	0.93	0.955	Heat-shock protein 16.9
BQ804592	0.226	1.381	1.623	0.375	0.303	Heat-shock protein 16.9
AF005993	0.244	4.703	2.597	0.934	0.844	Heat-shock protein, 70 kDa
CD896621	0.427	2.54	2.651	1.208	1.004	Heat-shock protein 16.9
BJ265952	0.494	3.097	7.348	2.077	3.081	Heat-shock cognate 70 kDa protein 1
Genes encoding ubiquitin-pathway related proteins						
BJ272261	0.083	22.42	5.645	1.273	1.031	Ubiquitin extension protein
BJ216982	0.264	5.101	4.426	1.349	1.163	Ubiquitin
BJ218583	0.391	2.504	2.351	0.93	0.968	Ubiquitin–protein ligase
CA703369	0.669	2.181	2.864	1.714	1.659	Ubiquitin-conjugating enzyme
BJ242344	0.719	2.863	2.584	2.089	1.825	Ubiquitin
BJ273013	0.861	3.112	2.907	2.758	2.421	Ubiquitin (tetraubiquitin, polyubiquitin)
CD936252	0.048	24.18	17.32	1.127	0.865	skp1 protein (E3 Ubiquitin ligase SCF complex subunit)

Table legends are as defined in Table 3.

Discussion

Although resistance to insects has been identified in various plant species and is used extensively for the management of insect pests (Panda and Khush 1995; Quisenberry and Clement 2002), understanding of molecular events that contribute to this resistance is limited. Among plant–insect interactions, the molecular aspects of tomato resistance to aphids mediated by the *Mi-1.2* gene are the most extensively studied (Milligan et al. 1998; Rossi et al. 1998; Nombela et al. 2003). *Mi-1.2* confers resistance to both insects (aphids and white flies) and nematodes, and it contains the NBS-LRR structural motif common to many *R* genes that confer resistance to various pathogens. The fact that *Mi-1.2* confers resistance to taxonomically different parasites and that its product shares structural similarity with disease *R* genes suggests that recognition and activation of plant defense in response to attacks from insects, nematodes, and pathogens has been conserved throughout evolution.

However, because of differences in how parasites attack plants and obtain their nutrition, plant strategies for defense must, to some degree, be tailored to the specific parasite. Although *Mi-1.2* confers resistance to both nematodes and aphids, resistance to nematodes is effective in all growth stages of the plant and is associated with induction of hypersensitive response (HR), whereas resistance to aphids exists only in mature plants and is not associated with HR (Goggin et al. 2004; Bhattarai et al. 2007). Moreover, in crops other than tomato, *Mi-1.2* transgenic plants exhibit resistance to nematodes but not to aphids (Goggin et al. 2006). Clearly, the downstream events that lead to aphid resistance are different from those leading to nematode resistance. This makes sense, as nematodes and aphids have very different ways of obtaining nutrients from the plant.

The wheat–HF interaction is similar to a number of plant–pathogen interactions in that it shows features of gene-for-gene interactions between plant *R* genes and insect *Avr* genes (Ratcliffe and Hatchett 1997; Lobo et al. 2006). HF larvae are also similar to many pathogens in that they appear to secrete various effector proteins into plant cells (Hatchett et al. 1990; Chen et al. 2004, 2006; Harris et al. 2006). These common features indicate that the defense response of wheat to HF attack follows a similar molecular process in signal perception and transduction. Thus, the *R* product is part of the plant's surveillance system (Nimchuk et al. 2003; Chisholm et al. 2006; Dodds et al. 2006; Garcia–Brugger et al. 2006) and confers resistance by allowing the plant to mount early and effective defense. Early defense is especially necessary when the parasite creates a feeding tissue within the plant and also actively suppresses defense responses. The former has been shown to be the case with the virulent HF larva, which induces a feeding tissue (Harris et al. 2006) that is associated with the suppression of growth in the seedling (Anderson and Harris 2006).

Induced Susceptibility Vs Induced Resistance Based on the extensive published literature on the wheat–HF interaction (reviewed in Berzonsky et al. 2003; Harris et al. 2003) and the results of this study, a model for wheat–HF interaction is proposed (Fig. 4). By using a specialized pattern of attack (Rohfritsch 1992), HF larvae inject various substances into plant cells (Chen et al. 2004, 2006) and, thereby, elicit a range of physiological changes in the plant. During compatible interactions, these changes lead to the activation of susceptibility-related transcription regulators, which up-regulate the expression of genes involved in the metabolism and transport of nutrients, and down-regulate genes involved in cell-wall metabolism and in the synthesis of phenylpropanoids and other defense molecules. The larva, therefore, induces the plant to provide enhanced nutrition and to

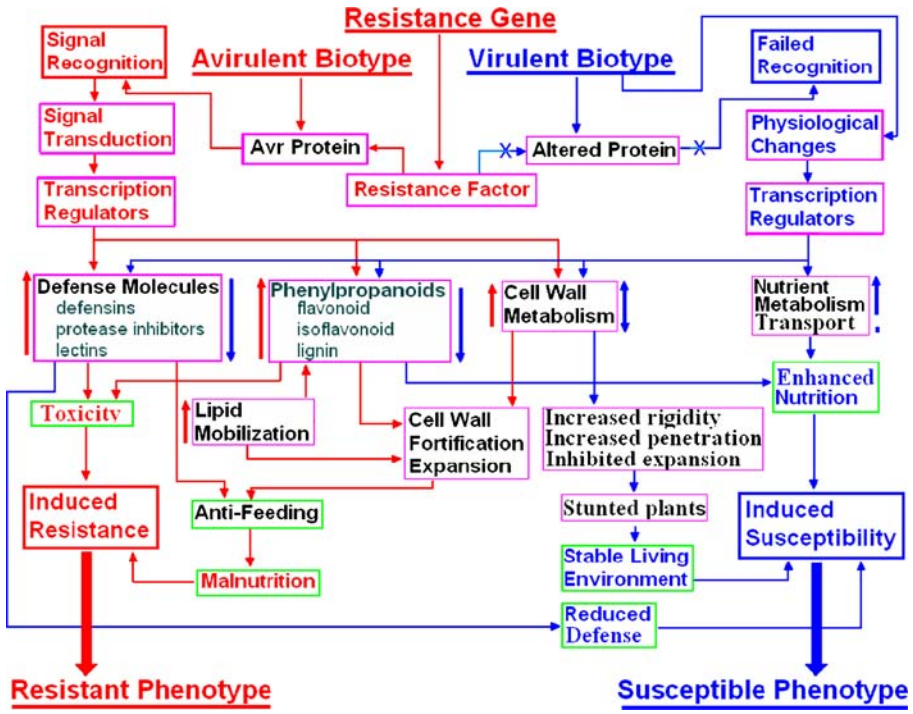


Fig. 4 A model for induced resistance vs induced susceptibility. During incompatible interactions, the recognition of an *avr* protein (produced by avirulent insects) by the resistance factor (encoded by an *R* gene) induces signal transduction and activates transcription, resulting in up-regulation (*arrow up*) of the pathways involved in antibiotic defense, phenylpropanoid synthesis, cell-wall expansion, and lipid mobilization. The up-regulation of these pathways results in increased toxicity and malnutrition, which constitute to host resistance. During compatible interactions, the resistance factor fails to recognize the (altered) protein produced by virulent insects. Instead, the invasion of the insect causes physiological changes in host plants, activating a different set of transcription regulators. These regulators up-regulate (*arrow up*) the pathways involved in nutrient metabolism, nutrient transport, and cell wall rigidity; and down-regulate (*arrow down*) the pathways involved in defense, phenylpropanoid synthesis, and cell-wall expansion. The combination of up- and down-regulation of these genes results in enhanced nutrition, stable living environment, and reduced defense, which contribute to host susceptibility

reduce exposure to plant defenses, i.e., the compatible interaction is actually a state of "induced susceptibility."

During incompatible interactions, the process begins when the wheat *R* protein detects HF attack by recognizing one or more of the secreted HF elicitors (*Avr* protein). This initiates a rapid defense response through a cascade of signal transduction, leading to the activation of defense-related transcription regulators. This, in turn, induces changes in gene expression in various pathways. As a result, the resistant plant prevents "induced susceptibility" by (1) not allowing the elevated production and transport of nutrients to the larval attack site, (2) producing plant defense chemicals, (3) fortifying the cell wall at the larval attack site, and (4) restoring normal plant growth. Taken together, these plant defense responses prevent the larva from obtaining nutrients from the plant, and the larva dies without changing in size. The prevention of susceptibility manipulation and enhanced defense constitute "induced resistance" during incompatible interactions.

Molecular Basis for Induced Susceptibility

Enhanced Nutrition During compatible interactions, enhanced larval nutrition results from better food quality and increased nutrient flow. Evidence for improved food quality came from the strong up-regulation of genes involved in nutrient metabolism, including genes involved in carbohydrate degradation such as α-glucosidase (BQ169475) and neutral invertase (BQ170874); genes in glycolysis and the citric acid cycle such as enolase (CK208852), pyruvate dehydrogenase (CD903633), and oxidoreductases (BE446498, BQ161195, CA657311); and genes in amino acid synthesis such as aspartate aminotransferase (CA653416) and methionine synthase (BJ250109) (Table 6). The up-regulation of the carbohydrate degradation enzymes indicated that more carbohydrates were degraded. The up-regulation of enzymes in glycolysis and the citric acid cycle, and in the pathways for amino acid synthesis indicated that some of the degraded carbohydrates were converted into intermediates for amino acid synthesis. This observation is consistent with the finding that HF attack causes dramatic shift from carbon-containing compounds to nitrogen-containing compounds during compatible interactions (Zhu et al. 2007). The increase in amino acids should result in better food quality for HF larvae.

Evidence for increased nutrient flow came from the strong up-regulation of genes that encode various transporters, including genes encoding general sugar transporters (CD931721, BJ320954, BJ275570), glucose transporters (CD892342, BJ231104), a myo-inositol transporter (BQ171988), ammonium transporters (BT009318, CA601803), and amino acid transporters (BJ275567, CA737017, CA638660, CK205233, BJ286275, BT009250; Table 6). The up-regulation of these transporters should facilitate nutrient flow from other parts of the plant to the HF feeding site.

Reduced Defense In contrast to the up-regulation of genes involved in nutrient metabolism and transport, genes involved in plant defense were down-regulated during compatible interactions (Tables 3 and 4). For example, five out of the seven protease inhibitor genes were significantly down-regulated during compatible interactions. Two of them (BJ316836 and BJ314399) were completely suppressed. Plant protease inhibitors are toxic to insect digestive systems (Murdock et al. 1988). HF larvae use various proteases including trypsins, chymotrypsins, and carboxypeptidases for food digestion (Zhu et al. 2005; Liu et al. 2006). The lower expression of protease inhibitor genes indicated weaker antibiotic defense.

Altered Phenylpropanoid Metabolism The strong down-regulation of many genes involved in the phenylpropanoid pathway during compatible interactions indicated that the phenylpropanoid pathway was crucial for induced susceptibility. However, the exact mechanism of phenylpropanoid-related susceptibility remains unknown. All of the PAL genes were highly up-regulated in both compatible and incompatible interactions (Table 3), indicating that more L-phenylalanine was converted to *trans*-cinnamic acid in both cases. But most of the downstream genes that convert cinnamates into other phenylpropanoids, including caffeic acid *O*-methyltransferases (COMT), a dirigent (DT), a chalcone synthase (CHS), a flavanone 3-hydroxylase (F3H), betanidin-5-*O*-glucosyltransferases (BOGT), flavonoid 7-*O*-methyltransferases (Fl-OMT), an isoflavone *O*-methyltransferase (IOMT), and an isoflavone reductase (IRT), responded differently to HF attack during compatible and incompatible interactions. These genes were down-regulated during compatible interactions, but they either were not affected or were up-regulated during incompatible interactions.

The combination of up-regulation of PAL and down-regulation of the downstream genes during compatible interactions indicated that there was more accumulation of cinnamic acid. If that was the case, it would be interesting to know the biological impact of cinnamic acid on HF larvae. One possibility for the accumulated cinnamic acid is to be converted into cinnamic acid derivatives via alternative pathways (Dixon et al. 2002). High concentration of cinnamic acid derivatives might benefit HF larvae because of their antioxidant activity (Natella et al. 1999). The down-regulation of the downstream genes also indicated lower concentration of other phenylpropanoids such as chalcone, flavonoids, isoflanoids, and lignin. Many of these phenylpropanoids may be toxic to insects. For example, isoorientin, an abundant flavonoid in wheat, is toxic to corn earworm larvae (Widstrom and Snook 1998). The lower concentration of toxic phenylpropanoids again indicated weaker antibiosis during compatible interactions.

Cell-Wall Weakening and Growth Inhibition Lower concentrations of some phenylpropanoids such as lignin could also have a negative impact on cell-wall fortification and expansion because they are also cell-wall components. By coincidence, genes that encode enzymes that are in the pathways for the biosynthesis of other cell wall components, such as cellulose (cellulose synthase) and wax (CER1), were also down-regulated during compatible interactions (Table 5). In addition, genes that encode enzymes involved in cell-wall loosening (a necessary process for cell-wall expansion, Cosgrove 2003), such as XET, β-expansin, endo-1,4-β glucanases, pectinesterase, and polygalacturase, were also down-regulated during compatible interactions. Lower expression of the genes in the biosynthetic pathways for cell-wall components and loosening were consistent with the inhibition of cell-wall fortification and expansion during compatible interactions. Weaker cell wall could facilitate insect feeding.

While lower expression of genes in the biosynthetic pathways for cell-wall components and loosening were consistent with the inhibition of cell wall fortification and expansion, there were, however, several genes that exhibited up-regulation during compatible interactions. The up-regulated genes included the genes that encode a pectin-glucuronyltransferase (pectin synthesis), a pectinacetylesterase (pectin deacetylation), two xyloglucan fucosyltransferases (hemicellulose xyloglucan branching), and an arabinofuranohydrolase (hemicellulose hydrolysis). The relevance of the up-regulation of these genes to the inhibition of cell-wall fortification and expansion remains to be elucidated. It is possible that an increase in pectin and hemicellulose content and in xyloglucan branching can increase the rigidity of the cell wall, thus, inhibiting cell-wall expansion. Phenotypically, the growth of host plants is irreversibly inhibited during compatible interactions, whereas the overall growth of host plants is not significantly affected during incompatible interactions (Byers and Gallun 1972; Anderson and Harris 2006).

Molecular Basis for Induced Resistance

Prevention of Elevated Production and Transport of Nutrients The mechanism for induced resistance mediated by *R* genes appears to be the prevention or reversal of events leading to susceptibility. The prevention of elevated production and transport of nutrients might be an important mechanism for *R* gene-mediated resistance. This could be seen from the fact that the genes involved in nutrient metabolism and transport exhibited much lower expression during incompatible interactions than that during compatible interactions (Table 7). The I/C ratio for the 12 nutrient metabolic genes ranged from 0.105 to 0.751, and the I/C ratio for the 14 transporter genes ranged from 0.04 to 0.704. Many

other nutrient metabolic genes that were not included in the microarray were also up-regulated strongly during compatible interactions, but were either not affected or down-regulated during incompatible interactions according to Northern blots that use probes derived from wheat-related species such as rice or corn (Zhu et al. 2007). The lower expression of genes involved in nutrient metabolism and transport could effectively limit nutrients available to HF larvae at the attacking site, thus, inhibiting larval growth and development.

Enhanced Antibiosis Evidence for increased defense in resistant plants came from the elevated expression of the genes that encode molecules toxic to insects. For example, several genes that encode protease inhibitors exhibited significant higher expression during incompatible interactions (Table 3).

In addition to protease inhibitors, a group of genes that encode lectins or lectin-like proteins, including *Hfr-1*, *Wci-1*, and *Hfr-3*, had been previously reported as up-regulated strongly during incompatible interactions, and, therefore, were proposed to be involved in wheat defense against HF (Williams et al. 2002; Subramanyam et al. 2006; Giovanini et al. 2007). Lectins have been also reported to have antifeedant effects on other insects (Moreno et al. 1990; Peumans and Van Damme 1995). In this research, five lectin or lectin-like genes, including the previously idenfied *Hfr-1*, were up-regulated strongly during incompatible interactions (Table 3). The strong up-regulation of these lectin genes indicated that they were likely to have played a role in wheat antibiotic defense.

Phenylpropanoids and other phytochemicals are also defense molecules produced by plants when under attack (Dixon et al. 2002). As mentioned earlier, the up-regulation of the PAL genes during both compatible and incompatible interactions indicated more conversion of L-phenylalanine to *trans*-cinnamate. During incompatible interactions, cinnamate might have been converted into chalcones, flavonoids, isoflavonoids, lignin, and other phenylpropanoids because many genes that encode downstream enzymes that convert cinnamates into these phenylpropanoids were up-regulated, including COMT, CHS, F3H, BOGT, Fl-OMT, and IOMT. Higher concentration of toxic phenylpropanoids during incompatible interactions should also enhance antibiosis.

Cell-Wall Fortification and Plant-Growth Restoration Cell-wall fortification plays a major role in plant resistance to pathogens (Hammond-Kosack and Jones 1996). Cell-wall fortification may also play a critical role in wheat resistance to HF larvae. Most of the genes involved in biosynthesis of cellulose (cellulose synthase), wax (CER1), and lignin (dirigent), and the genes involved in cell-wall loosening and expansion, including xyloglucan endotransglycosylases, β-expansins, pectinesterases, and glucanases, were up-regulated strongly during incompatible interactions (Table 5). The up-regulation of these genes is favorable for cell-wall fortification and expansion. The fortification of the cell wall could play a role in anti-feeding by inhibiting HF larvae to establish feeding sites. The continuous expansion of cell wall might accelerate the restoration of plant growth after some initial growth inhibition during incompatible interactions (Anderson and Harris 2006).

Other Changes during Compatible and Incompatible Interactions

Histones and Other Cellular Structural Proteins The genes that encode various histones and a histone acetyltransferase were consistently down-regulated during compatible

interactions. The down-regulation of the histone genes could be a result of reduced cell division because the levels of histone mRNAs are believed to be regulated through rapid degradation in non-dividing cells (Kaygun and Marzluff 2005). Alternatively, the lower expression levels of histone genes could be caused by the differentiation of nutritive cells (Harris et al. 2006). Whether the down-regulation of the histone genes is the cause or the result of wheat growth inhibition during the compatible interactions will have to be determined in future experiments. Although the genes that encode histones (the chromosomal structural proteins) were down-regulated during compatible interactions, the genes that encode cellular structural proteins including actins and α-tubulin (usually up-regulated in growing tissues) were significantly up-regulated during compatible interactions. The growth of susceptible plants was completely inhibited during compatible interactions. While evidence suggested that actins and tubulins may be involved in plant defense against pathogens (Kobayashi et al. 1997; Kobayashi and Hakuno 2003), the biological significance of up-regulation of actin- and tubulin-encoding genes during compatible interactions in our experiments remains to be resolved. One possibility is that more actins and tubulins are needed to fortify the cells mechanically in non-dividing tissues to withstand mechanical damages caused by HF larvae.

Greater Stress Response During Compatible Interactions In terms of gene regulation, there was much greater stress response to HF infestation during compatible interactions than during incompatible interactions. Many genes that encode various heat-shock proteins were dramatically up-regulated during compatible interactions, whereas these genes were not affected or even down-regulated during incompatible interactions (Table 8). A similar phenomenon was also observed with several genes that encode ubiquitins or ubiquitin-pathway enzymes. In addition, many other genes that encode other stress proteins, such as dehydrins, wound-inducible proteins, and cold-responsive proteins, were also dramatically up-regulated during compatible interactions. Again, these stress genes, with only a few exceptions, were not significantly affected during incompatible interactions. The greater stress response during compatible interactions may simply be a reflection of greater stresses upon susceptible plants, which were completely taken over by the HF larvae with plant metabolism totally redirected to feed the larvae (Anderson and Harris 2006; Harris et al. 2006). Some of the stress genes might also be linked with host susceptibility. Many of the known host plant *R* genes in wheat are temperature-sensitive. Resistant wheat plants that carry a temperature-sensitive *R* gene become susceptible at higher temperature. Whether there is a relationship between these stress proteins and temperature sensitivity of host-plant resistance will need further investigation.

Acknowledgment This work was supported by two grants from the National Research Initiative Competitive Grant program, the US Department of Agriculture (USDA 04-35607-14861 and USDA2005-35302-16254). This paper is contribution no. 07-81-J from the Kansas Agricultural Experiment Station. Hessian fly voucher specimens (no. 150) are located in the KSU Museum of Entomological and Prairie Arthropod Research, Kansas State University, Manhattan, Kansas. The microarray chips were processed with the Affymetrix GCS 3000 system in the Gene Expression Facility, Kansas State University. The system was purchased through the NSF Major Research Instrumentation grant DBI-0421427. The authors thank Dr. Subbaratnam Muthukrishnan, Dr. Guihua Bai, and Dr. Scot Hulbert for reviewing an earlier version of the manuscript. Mention of commercial or proprietary product does not constitute endorsement by the USDA.

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considering more than one generation. Thus, cyanide-containing precursors can have negative effects even on herbivores adapted to feed on cyanogenic plants.

Keywords Herbivory · Chemical defense · Plant–herbivore interaction · Generalist · Specialist · Lima bean · *Phaseolus lunatus* · Cyanogenic glycosides · Mexican bean beetle · *Epilachna varivestis*

Introduction

Plant cyanogenesis is considered a direct plant defense against herbivores and means the release of hydrogen cyanide (HCN) from damaged plant tissues (Poulton 1990). More than 2,500 species of vascular plant taxa belonging to over 130 families are cyanogenic (Møller and Seigler 1999). As HCN is toxic to the plant itself, nontoxic cyanide-containing precursors are synthesized and stored in compartments, spatially separated from specific enzymes (β-glucosidases and α-hydroxynitrile lyases) required for their degradation and the following release of HCN (Selmar et al. 1989). In lima bean, the glycosidic precursors linamarin and lotaustralin accumulate in the vacuoles (Frehner and Conn 1987). Specific β-glucosidases are located in the apoplast, whereas α-hydroxynitrile lyases are membrane-associated (Conn 1981; Selmar et al. 1987, 1989).

The release of HCN occurs only in disintegrated tissue. Although cyanogenesis is generally regarded as a constitutive mechanism, recent studies on lima bean have demonstrated an induced component: β-glucosidases involved in the release of HCN from cyanide-containing compounds were activated in response to herbivore damage (Ballhorn et al. 2006).

There is increasing evidence that plant cyanogenesis or cyanogenic glucosides deter generalist, but not specialist, herbivores (Jones 1988; Patton et al. 1997; Ballhorn et al. 2005). Strongly cyanogenic plants are protected from generalists (Viette et al. 2000; Ballhorn et al. 2005) but nevertheless can be attacked by specialists (Benson et al. 1976; Davis and Nahrstedt 1987). Generally, specialist or oligophagous herbivores with restricted host ranges are able to tolerate or to detoxify defensive compounds of their respective host plants because of physiological or behavioral adaptations (Rausher 1996; Agrawal and Kurashige 2003; Agrawal and Van Zandt 2003). In some cases, they even use the toxic plant compounds for their own defense (Bowers and Stamp 1997; Dobler et al. 1998), and other cases are known, in which cyanogenic glycosides act as phagostimulants for herbivores adapted to cyanogenic plants (Lapidus et al. 1963).

Studies conducted so far on specialists that live on cyanogenic plants have shown that, in principle, they can develop on such plants (Beesley et al. 1985; Engler et al. 2000; Urbańska et al. 2002). Nevertheless, whether or not specialists are quantitatively affected by cyanogenic features of their hosts remains elusive (Miguel and Alberto 2005). Long-term studies are lacking on the quantitative dependence of parameters relevant to fitness, such as growth rates, developmental period, and success of reproduction itself, as quantitatively depending on cyanogenic features of host plants.

The present study was conducted to fill this gap in functional understanding of distinct cyanogenic components. We studied quantitative effects of cyanogenic precursor concentration (HCNp, cyanogenic potential) and cyanogenic capacity (HCNc; release of gaseous HCN per unit time) on Mexican bean beetle (Coleoptera: Coccinellidae: *Epilachna varivestis* Mulsant).

We analyzed several fitness-relevant parameters over four consecutive generations of beetles (the performance of all four larval stages, the duration of pre-pupation and pupal

stages, and the body mass at emergence, and total reproduction) in different experimental settings by using selected plants with well-defined cyanogenic properties (Ballhorn et al. 2005, 2006, Ballhorn and Lieberei 2006). Based on significant differences in their HCNp, we grouped lima bean accessions into high cyanogenic (HC) and low cyanogenic (LC) plants. This set of experimental plants allowed us to quantify effects of plant cyanogenic features on the performance of beetles.

Materials and Methods

Plants and Insects The lima bean (Fabaceae: *Phaseolus lunatus* L.) is an obligate cyanogenic plant of Mesoamerican and Andean origin (Baudoin et al. 1991). We used lima beans of 12 accessions (11 domesticated lines and 1 wild-type native to Cuba) characterized by different cyanogenic features. Seeds were provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany. For further description of plant material, see Ballhorn et al. (2006). In addition, snap bean *Phaseolus vulgaris* cv. Saxa (Carl Sperling, Lüneburg, Germany, stock number 40.176), hereafter referred to as "Saxa," was used as a non-cyanogenic control.

The Mexican bean beetle (Coccinellidae: *E. varivestis* Muls.) is an oligophagous insect that feeds on a range of legumes (Barrigossi et al. 2001) but with distinct preference for *Phaseolus* species (Dover et al. 1988) and especially lima bean (Flanders 1984; Baudoin et al. 1991). As feeding of the beetle is stimulated by cyanogenic glycosides even in artificial diets, an adaptation to diets containing these compounds can be assumed (Lapidus et al. 1963). Mexican bean beetle is native to southern Mexico as are lima beans of the Mesoamerican gene pool. The life cycle of the beetles encompasses four larval stages followed by a short pupal stage of 5–10 days. Females deposit eggs in clutches of 40–75 on the lower surface of host plant leaves, and both larvae and adults feed on foliage.

Rearing of Plants and Insects Plants were cultivated under greenhouse conditions (16:8 L/D with a photon flux density of 350–450 μmol s^{-1} m^{-2} at the plant container and 850–950 μmol s^{-1} m^{-2} on top of the plants depending on natural radiation). Additional light was provided by 400 W high-pressure sodium lamps (Son-Targo 400, Philips®). To avoid effects by hot spots under the lamps, position of plants was changed every 3 days. Temperature (25/20°C) and ambient relative air humidity (60–70%) were controlled by Intergro® software. Plants were fertilized with a nitrogen–phosphate fertilizer (Blaukorn®-Nitrophoska®-Perfekt, Compo GmbH) twice a week (3 mg per pot) and were cultivated in plant containers 18 cm diameter in a 1:1 ratio of standard substrate (TKS®-1-Instant, Floragard®) and sand (grain size 0.5 – 2.0 mm). Plants (N=12 per accession) were grown from seeds obtained by self-fertilization of a single mother plant.

Beetles were maintained on "Saxa" to prevent them from developing preferences for any particular *P. lunatus* accession. In addition, large numbers of beetles (>250 per accession) were maintained on plants of all 12 lima bean accessions used in the feeding trials. Ambient conditions adjusted for both maintenance cultures of insects and feeding experiments were identical.

Analysis of Leaf Quality Individual leaves of all accessions were used for analysis of cyanogenic features (HCNp, β-glucosidase activity, and HCNc) and nutritive traits (soluble

protein and total phenolics). Young trifoliate leaves (N=12 leaves per accession, N=1 leaf per plant) fully unfolded for 5–7 days were selected for the experiments as described in Ballhorn et al. (2006). One randomly selected leaflet of each trifoliate leaf was cut along the midrib. One half was used for HCNp analysis, the other half for the determination of soluble protein concentration and β-glucosidase activity. The remaining two leaflets were used for quantification of HCNc and total phenolics.

Cyanogenic potential (HCNp) was analyzed by complete enzymatic degradation of cyanogenic glycosides, and HCN released from cyanogenic precursors was spectrophotometrically quantified (585 nm) by using the Spectroquant® cyanide test (Merck, Darmstadt, Germany) following Ballhorn et al. (2005). External β-glucosidase isolated from rubber tree (Euphorbiaceae: *Hevea brasiliensis*) was added to ensure complete degradation of cyanogenic precursors. This enzyme showed high affinity to cyanogenic glycosides in lima bean and was isolated following Ballhorn et al. (2006).

Cyanogenic capacity (HCNc) was measured as gaseous HCN released into the atmosphere per unit time after leaves were treated with chloroform. We used an air-flow system (7 l air min^{-1}) for detection of the HCNc as described in Ballhorn et al. (2005).

Soluble protein concentration was quantified according to Bradford (1976). Leaf material was homogenized in ice-cold sodium acetate buffer (pH 5.0). Leaf extracts were centrifuged (Heraeus®Biofuge Fresco, 13,000 rpm, 4°C), and the supernatant was filtered over NAP™ columns containing Sephadex™ G-25 DNA-Grade (GE Healthcare Bio-Sciences AB). Subsequently, 5 µl of the eluate were pipetted on microplates (96-well Microplates, PS, F-bottom, Greiner Bio-One GmbH, Maybachstr. 2, 72638 Frickenhausen, Germany), and 250 µl Bradford reagent (diluted with aqua dest. in the ratio 1:4) were added. Protein concentration of samples was spectrophotometrically quantified at 595 nm (Spectra MAX 190 PC, Molecular Devices). BSA solutions (Merck, Darmstadt, Germany) in the range between 10 and 300 µg ml^{-1} served as standard.

Activity of β-glucosidase was measured according to Ballhorn et al. (2006). We used *p*-NP-glucoside (Merck) as chromogenic artificial substrate. The released *p*-nitrophenol was quantified spectrophotometrically at 400 nm (Pharmacia Biotech, Ultraspec 3000).

Total phenolics were analyzed following Tikkanen and Julkunen-Tiitto (2003). Homogenates of leaves were extracted three times for 15 min while stirring in 50-ml acetone diluted with aqua dest. (60:40). After each extraction, samples were incubated in an ultra sonic bath (3 min) and were finally centrifuged for 10 min at 5,000×g. The supernatant was transferred in 2-ml concentrated acetic acid (Merck), acetone was removed in vacuum (60 mbar) at 40°C, and the residue was quantitatively transferred by using aqua dest. Samples were diluted with 2.5% acetic acid, and 1 ml of this solution was mixed with 0.5 ml Folin–Ciocalteus phenol reagent (Merck). After adding 2 ml 20% Na_2CO_3, the solution was made up to 10 ml with aqua dest. Samples were incubated at 70°C and, after cooling, spectrophotometrically quantified at 730 nm against a blank containing water instead of sample. Epicatechine (Sigma, Deisenhofen, Germany) served as standard.

Feeding Trials Beetles' performance on leaves of *P. lunatus* accessions was analyzed in no-choice long-term feeding experiments. Males and females were considered separately due to inter-sex variability in weight gain. These experiments included four consecutive generations. Freshly hatched larvae (N=11 per generation) were individually placed on single detached leaves supplied with water (perforated Eppendorf tubes). Feeding trials were carried out in petri dishes (9.5 cm) lined with slightly moist filter paper to avoid wilting of leaf material under controlled conditions (25/20°C, photon flux density of

200 μmol s^{-1} m^{-2} in a 16:8 L/D period). Leaf material was renewed daily, and larvae were weighed daily until pupation. At the end of the assays, body weight and sex of the freshly emerged beetles were documented.

In these long-term experiments, trans-generational effects were also considered. The reproduction of beetles on different bean accessions was analyzed by pooling freshly emerged female beetles in a cage that contained four intact plants of the respective accession. Male beetles that emerged in the same experiment were immediately exchanged against freshly emerged males from large maintenance populations of Mexican bean beetles (>250 beetles per accession). These populations were kept for the same time period on the same lima bean accessions that were used in the feeding trials. These "external" males were added to the females in a 1:1 sex ratio. Number of males added depended on number of females that emerged in the experiment. By this procedure, we avoided creating a family bias on the different accessions, which otherwise might have led to artificial effects of development of different beetle genotypes. Eggs produced by the beetles were removed from plants by cutting leaf discs around the attached egg clutches. Eggs were incubated until hatching under identical ambient conditions that were adjusted for long-term feeding trials. The number of eggs and of hatched larvae was documented for all egg clutches. Because females started oviposition simultaneously and there were constant intervals of 8–9 days between productions of egg clutches per female, these clutches were assigned as consecutive clutches (1, 2, and 3) in this pooled experimental setup. A part of the progeny (N=11 randomly selected larvae per accession) was used for the next generation again, which was studied under the same experimental conditions as mentioned above.

In these long-term experiments, detached leaves were used. Detached leaves are sensitive to changes in leaf biochemistry depending on degradation processes of cell components. These processes can lead to a spontaneous release of gaseous HCN from leaf material, which complicates quantitative analysis of cyanogenesis and its impact on herbivore performance. Therefore, to test for losses of HCN during the experimental period, water-supplied leaves were placed in an air-flow system according to Ballhorn et al. (2005) under ambient conditions identical to those used for feeding experiments, and released HCN from leaf material was measured quantitatively. No loss of HCN from leaves was detected.

Larval Efficiency of Food Utilization We calculated three parameters of food utilization efficiency (Farrar et al. 1989; Anaya et al. 2003; Jallow et al. 2003). Relative growth rate (RGR) was calculated as: biomass gained (mg fresh weight)/[duration of feeding period (days) × mean fresh weight (mg) of larvae during feeding period]. The relative consumption rate (RCR) estimates the weight of food consumed relative to time and mean weight of the insect during the feeding period and was calculated as: food ingested (mg dry weight)/[duration of feeding period (days) × mean fresh weight (mg) of larvae during feeding period]. The efficiency of conversion of ingested food (ECI) estimates the percentage of ingested food that is converted to biomass. It was calculated as: [biomass gained (mg fresh weight)/food ingested (mg dry weight)] × 100.

The experiments were carried out in Petri dishes (9.5 cm). We used larvae of the third instars between days 6 and nine from hatching and leaf discs (2.73 mm diameter), which were exchanged daily. Two discs per setup were cut from two randomly selected leaflets of one trifoliate leaf, each from one leaflet. Positions on the lamina at which the discs were removed were the same for both leaflets. One disc was used for the feeding trial; the other was dried at 45°C to constant weight for determination of dry matter and leaf mass per unit

leaf area (LMA). Leaf discs cut for the feeding trials were put on moist filter paper and then were placed in the air-flow system according to Ballhorn et al. (2005) before the experiment. Leaf discs were used for feeding trials when no more gaseous HCN was released from the cutting edge (5–10 min after cutting). Release of gaseous HCN from leaf discs by wilting during the feeding trial was avoided by placing leaf discs on moist filter paper in the Petri dishes. We tested for spontaneous loss of HCN over the experimental period from leaf discs in the same way as described for long-term feeding trials with intact detached leaves.

After the feeding trial, remaining leaf material was dried at 45°C and weighed to the nearest 0.001 mg. Dry weight equivalents of food consumed by individual larvae were obtained by multiplying the daily wet weight of food consumed by percentage dry matter in each diet (Waldbauer 1968). The remaining third leaflet of the leaf was used for analysis of cyanogenic precursor concentration (HCNp) to check for constancy in precursor concentrations of leaves actually used in feeding trials, as compared to leaves that were used for analysis of leaf quality of the respective accessions before.

Statistical Analysis Differences in HCNp, HCNc, and nutritive components, i.e., soluble protein concentration and total phenolics among lima bean accessions and differences in beetles' performance on different accessions, were analyzed by one-way analysis of variance (ANOVA) and *post hoc* comparisons (HSD). The effects that different accessions of lima bean, i.e., plants with different HCNp, had on egg production were consequently analyzed with a repeated measures ANOVA design on a balanced subset of data created by randomly selecting four females per generation/accession/clutch number-combination after testing the assumption of sphericity with Mauchly's test. Data comprised four consecutive generations of beetles and three consecutively laid egg clutches per generation. Statistical analyses were carried out by use of Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS for Windows, SPSS, Chicago, IL, USA).

Results

Leaf Quality Lima bean accessions showed distinct differences in leaf HCNp (Table 1). The three accessions with the highest (high cyanogenic, HC) and the lowest concentration of cyanogenic precursors (low cyanogenic, LC) were used (Table 1).

The difference in HCNp between HC- and LC-plants was significant (one-way ANOVA; $F_{173}=500.57$, $P<0.001$). In addition to HCNp, HCNc was highly variable among the accessions that were used for feeding experiments, depending on cyanogenic precursor concentration and activity of specific endogenous β-glucosidases. For instance, accession 2116 was characterized by the highest concentration of cyanogenic precursors, yet showed low HCNc due to a low activity of β-glucosidase (Table 1).

In contrast to cyanogenic features, total phenolics and concentration of soluble protein in leaves and LMA did not differ significantly among the accessions (Table 1).

Body Mass of Beetles Substantial inter-sex variability in beetles' body mass was observed among HC- and LC-plants. Females showed higher body mass compared to males independent of plant cyanogenic features (HC: $F_{1,12}=102.56$, $P<0.001$; LC: $F_{1,13}=82.18$, $P<0.001$; "Saxa": $F_{1,44}=69.31$, $P<0.001$). Thus, male and female beetles were considered

Table 1 Cyanogenic and nutritive traits of leaves

Cyanogenic Traits					Nutritive Traits		
Accessions	Cyanogenic Status [High (HC) and Low (LC)]	Cyanogenic Potential HCNp (μmol HCN g^{-1} fw)	Cyanogenic Capacity HCNc (μmol HCN g^{-1} fw min^{-1})	β-Glucosidase Activity (μkat g^{-1} dw)	Total Phenolics (mg g^{-1} dw)	Soluble Protein (mg g^{-1} dw)	Leaf Mass per Area LMA (mg cm^2)[a]
2116[b]	HC	76.5±15.3 (a)	0.10±0.03 (g)	0.06±0.04 (h)	1.92±0.30 (a)	140.55±8.70 (a)	0.80±0.11 (a)
2357[b]	HC	68.4±14.8 (ab)	0.58±0.07 (ab)	1.91±0.26 (b)	2.11±0.41 (a)	140.04±2.59 (a)	0.80±0.12 (a)
WT[b,c]	HC	64.7±11.4 (ab)	0.64±0.07 (a)	2.85±0.33 (a)	2.34±0.42 (ab)	144.07±4.12 (a)	0.83±0.13 (a)
8078	–	61.8±6.0 (ab)	0.56±0.05 (bc)	1.90±0.22 (bc)	2.18±0.41 (ab)	139.80±6.91 (a)	0.81±0.09 (a)
1315	–	55.9±11.4 (bc)	0.49±0.08 (c)	1.64±0.25 (cd)	2.40±0.40 (ab)	144.23±3.95 (a)	0.79±0.10 (a)
1738	–	40.1±12.7 (cd)	0.29±0.08 (d)	1.42±0.23 (def)	2.21±0.53 (ab)	137.31±12.64 (a)	0.82±0.11 (a)
8151	–	35.0±5.9 (de)	0.27±0.06 (de)	1.34±0.18 (ef)	2.32±0.51 (ab)	140.77±2.60 (a)	0.82±0.09 (a)
8073	–	28.6±7.2 (def)	0.20±0.06 (ef)	1.53±0.25 (de)	2.72±0.50 (ab)	137.24±5.41 (a)	0.81±0.12 (a)
2441	–	19.8±4.8 (efg)	0.08±0.01 (g)	0.07±0.04 (h)	1.94±0.24 (a)	141.16±2.51 (a)	0.80±0.07 (a)
8067[b]	LC	18.5±4.2 (fg)	0.15±0.06 (fg)	1.25±0.11 (fg)	2.53±0.69 (ab)	138.50±9.93 (a)	0.81±0.10 (a)
8079[b]	LC	9.3±3.2 (fgh)	0.14±0.03 (fg)	1.01±0.12 (g)	2.64±0.41 (ab)	142.95±3.71 (a)	0.80±0.12 (a)
8071[b]	LC	7.3±2.1 (gh)	0.13±0.04 (g)	1.22±0.15 (fg)	2.81±0.42 (ab)	140.41±5.79 (a)	0.82±0.10 (a)
Saxa[b,d]	Non-cyanogenic	0.0±0.0 (h)	0.00±0.00 (h)	0.01±0.01 (h)	3.16±0.22 (b)	142.02±4.27 (a)	0.83±0.10 (a)

[a] Values shown for leaf characteristics are means (±SD; N=12 individual plants per accessions). Significant differences between accessions were calculated by a *post hoc* test (Tukey's HSD; $P<0.001$) after one-way ANOVA and are indicated by different letters in parentheses.

[b] Accessions used for feeding trials (data for HCNp presented also in Fig. 1)

[c] Accession WT represents lima bean, *P. lunatus*, wild type (Cuba) with small seeds and leaves.

[d] Saxa represents *P. vulgaris* cv. Saxa

separately (Fig. 1). Male and female adults had significantly lower body mass at emergence when larvae had developed on HC-plants as compared to LC-plants and "Saxa", whereas beetles that had developed on LC-plants showed no significant differences in body mass compared to beetles whose larvae had fed on "Saxa" (Fig. 1). Body mass of male and female beetles on plants of the same accessions did not vary significantly among four consecutive generations. In contrast to clear effects of high HCNp on beetles' body mass, variation of HCNc in the group of high HCNp plants had no significant effect on adult weight. Beetles feeding on the accession 2116 (high HCNp, low HCNc) showed no differences in body mass compared to the other two HC-plants, accession 2357, and to lima bean wildtype (WT), which were characterized by high HCNp and HCNc (males: $F_{2.611}=2.23$, $P=0.117$; females: $F_{2.512}=0.734$, $P=0.485$).

Development of Beetles The developmental period of individual beetles was affected quantitatively by HCNp concentrations. Among HC-plants, larval feeding period, pre-pupation phase (larvae attached to leaves but not yet pupated), and pupal dormancy were significantly prolonged on HC-plants as compared to LC-plants and non-cyanogenic "Saxa" (Table 2). We found no significant differences in phase length between LC-plants and "Saxa" in any of the beetles' life cycle phases. In contrast to strong quantitative effects of HCNp, no difference in performance and reproduction of beetles among HC-plants depending on quantitative variation of HCNc were observed. Females produced at least three egg clutches in intervals of 8–9 days. Females kept laying eggs after 27 days, but these eggs were often deformed and were not positioned in distinct clutches. Thus, only the first three egg clutches were included in analyses of reproduction.

Egg clutch sizes, i.e., the numbers of eggs per clutch, significantly depended on generation and clutch number and on plant accession (Table 3). Interactions between all these factors indicate that the intergenerational effect depended on the accession on which beetles developed and that the strength of the intergenerational effect was different among the consecutively produced egg clusters (Table 3). In fact, the differences between HC- and LC-plants were already visible in the first generation but became more prominent in the following generations (Fig. 2). In particular, the third clutches became consecutively smaller in beetles reared on HC-plants, whereas no such effect was visible in beetles reared on LC-plants, or "Saxa". Consequently, *post hoc* analysis (LSD) grouped the three HC-accessions in one group that was significantly different from the three LC-accessions and

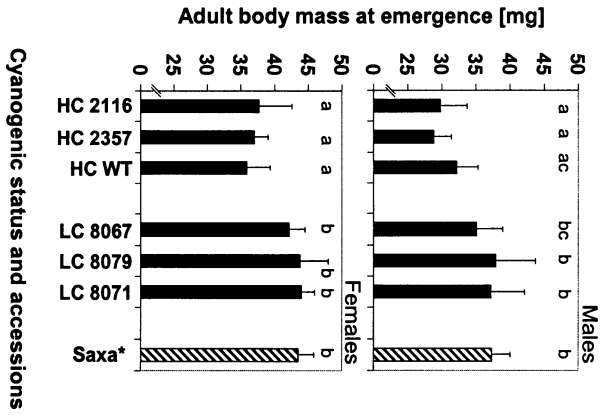


Fig. 1 Adult body mass at emergence. Body mass of male and female beetles, which had developed on lima beans with different HCNp (*HC* high cyanogenic, *LC* low cyanogenic, see Table 1) was measured. Values are means (±SD) among beetles of four consecutive generations (for number of replications see Table 2). Significant differences between groups were calculated by a *post hoc* test (Tukey's HSD; $P<0.001$) following one-way ANOVA and are indicated by different letters. Saxa* (= *P. vulgaris*), no HCN detected

Table 2 Effects of host plant cyanogenic status on developmental phases in beetles' life cycle

Accessions	Cyanogenic Status	Sex	*N*	Larval Feeding Period (day)	Pre-pupation Period (d)	Pupal Dormancy (d)
2116	HC	♂	23	18.1 ± 1.0^{ab}	3.0 ± 1.0^{a}	8.3 ± 0.8^{b}
2357	HC	♂	27	19.0 ± 0.4^{a}	3.1 ± 0.3^{a}	7.4 ± 0.8^{ab}
WT	HC	♂	25	19.0 ± 1.1^{a}	2.8 ± 0.7^{ab}	7.5 ± 1.1^{ab}
8067	LC	♂	22	17.5 ± 1.0^{bc}	1.8 ± 0.6^{c}	6.3 ± 0.6^{de}
8079	LC	♂	20	16.9 ± 0.9^{bc}	2.0 ± 0.4^{bc}	5.6 ± 0.8^{cd}
8071	LC	♂	22	16.6 ± 1.0^{c}	2.2 ± 0.6^{bc}	5.3 ± 0.5^{c}
Saxa	Non-cyanogenic	♂	21	16.8 ± 1.0^{c}	2.0 ± 0.6^{c}	6.7 ± 0.7^{ae}
2116	HC	♀	21	18.1 ± 0.6^{a}	3.0 ± 0.4^{a}	8.1 ± 0.6^{a}
2357	HC	♀	15	18.6 ± 0.5^{a}	3.1 ± 0.4^{a}	6.7 ± 0.5^{b}
WT	HC	♀	18	18.8 ± 1.1^{a}	2.5 ± 0.6^{ab}	7.8 ± 0.9^{a}
8067	LC	♀	21	17.9 ± 1.1^{ac}	2.0 ± 0.7^{b}	6.1 ± 0.8^{bc}
8079	LC	♀	24	17.0 ± 0.6^{bc}	1.9 ± 0.5^{b}	5.6 ± 0.6^{c}
8071	LC	♀	20	16.4 ± 0.9^{b}	2.0 ± 0.6^{b}	5.3 ± 0.7^{c}
Saxa	Non-cyanogenic	♀	22	17.1 ± 0.5^{bc}	2.2 ± 0.4^{b}	6.5 ± 0.6^{b}

Differences in performance of beetles depending on host plant accessions characterized by different HCNp (*HC* high cyanogenic, *LC* low cyanogenic) were tested using a *post hoc* test (Tukey's HSD) following one-way ANOVA. Different letters indicate significant differences in developmental period among beetles of four consecutive generations ($P<0.001$). Values shown for developmental periods are means (±SD).

"Saxa". Over four generations, larval hatching was also negatively affected by high host plant HCNp (Fig. 3).

Efficiency of Larval Food Utilization Indices of food utilization differed significantly among beetles on beans with different HCNp (Table 4). Larvae reared on HC-plants ($N=33$ larvae) showed an increased consumption rate and a reduced ECI into insect biomass compared to larvae reared on LC-plants (RCR: $F_{1,63}=19.126$, $P<0.001$; ECI: $F_{1,63}=83.820$, $P<0.001$; $N=55$). The RGR was reduced on HC-plants compared to LC-plants ($F_{1,63}=117.67$, $P<0.001$). We found no significant differences in all three parameters between the LC-plants and "Saxa" ($N=11$ larvae). The variation of HCNc in the group of HC-plants had no effect on beetles' food utilization.

Table 3 Intergenerational effects on clutch size

	Sum of Squares	*df*	*F*	*P* value
Within-subject effects				
gen	2,513.390	3	9.557	<0.001
gen × acc	7,205.881	18	4.567	<0.001
clu	39,629.720	2	160.523	<0.001
clu × acc	3,381.113	12	2.368	0.020
gen × clu × acc	3,829.994	6	5.760	0.000
	8,924.173	36	2.240	0.001
Between-subject effects				
Acc	15,087.619	6	9.262	<0.001

Effects of generation (*gen*) and clutch number (*clu*) as well as accession (*acc*) on clutch size (i.e., number of eggs per clutch) were tested by using a repeated measures ANOVA after confirming that there was no significant deviation from sphericity (tested with Mauchly's test). Four consecutive generations and three clutches per female were considered and generation and clutch were therefore included as within-subject variables, whereas lima bean accession served as between-subject variable.



Fig. 2 Eggs per clutch. Female beetles produced three egg clutches in each of the reproductive cycles between the feeding trials [total number of clutches per generation (gen): *gen 1* N=120; *gen 2* N=99; *gen 3* N=96; *gen 4* N=108]. Missing clutches were considered as 0. Values shown for number of eggs per clutch are means. For statistical analyses of effects on clutch size (i.e., number of eggs per clutch) such as beetle generation, clutch number, and accession of host plants see Table 3. Plants with different cyanogenic status were used for the experiments (*HC* high cyanogenic, *LC* low cyanogenic). Saxa* (= *P. vulgaris*), no HCN detected

Discussion

Defenses mediated by secondary plant compounds are generally believed not to affect specialist herbivores due to their capacity to tolerate or to detoxify defensive compounds of their host plants by behavioral or physiological adaptations (Rausher 1996; Zagrobelny et al. 2004). The specialist herbivore paradigm predicts that adapted herbivores—including oligophagous herbivores with restricted host range—are less affected by a given chemical defense as compared to generalists (Van Der Meijden 1996; Van Dam et al. 2000). However, the observation that a herbivore can develop successfully on a given host plant—or even that it prefers this particular host plant under artificial or natural conditions—does not necessarily mean that it is not affected negatively by some of its host plant's chemical defenses at higher thresholds, which actually occur in nature (Agrawal and Kurashige 2003).

To study such effects, quantitatively, we used an oligophagous herbivore, the Mexican bean beetle (*E. varivestis*), and reared it on different accessions of cyanogenic lima bean (*P. lunatus*), its preferred natural host plant species. The plants significantly differed in their cyanogenetic traits: Three accessions were characterized by concentrations of 64.7 to 76.5 μmol HCN g^{-1} fw (high-cyanogenic, HC) bound in cyanogenic precursors, whereas the "low cyanogenic" (LC) accessions had 7.3 to 18.5 μmol HCN g^{-1} fw.

In spite of its specialization on lima bean, concentration of cyanide-containing compounds significantly affected several fitness-relevant traits of the beetles. Larvae feeding on HC-leaves showed prolonged developmental times (Table 2) and gained less weight per time, although food intake rates were even higher on HC- than on LC-leaves (Table 4). Consequently, the conversion efficiency was lower on HC- than on LC-leaves (Table 4). As longer developmental times were not sufficient to compensate for lower growth rates, the average weight of adult beetles was lower on HC- than on LC-leaves (Fig. 1). Although male and female beetles differed in weight gain, both a reduced total body weight and a lowered larval and pupal performance were significant for both sexes.

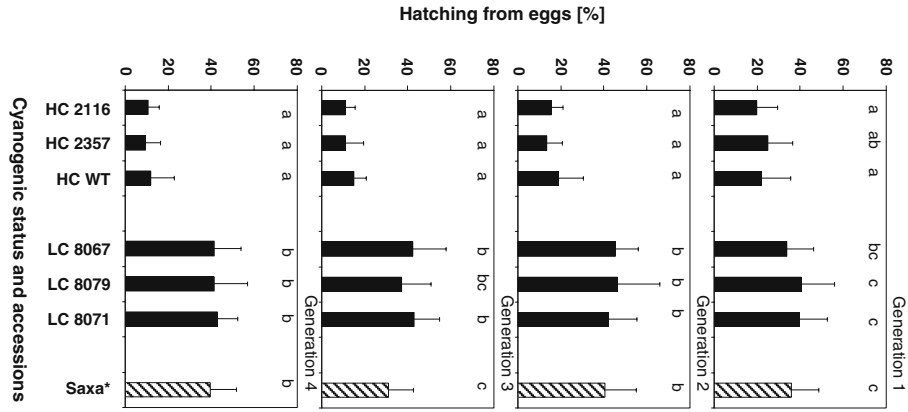


Fig. 3 Number of larvae per female beetle and generation. Hatching of larvae on different lima bean accession was observed over four consecutive generations of beetles. Values are means (±SD) of percentage hatching of larvae including eggs from all three clutches laid per female (N=141 female beetles). Significant differences between groups were calculated by a *post hoc* test (Fishers LSD; P<0.001) following one-way ANOVA and are indicated by different letters. Plants with different cyanogenic status were used for the experiments (*HC* high cyanogenic, *LC* low cyanogenic). Saxa* (= *P. vulgaris*), no HCN detected

The effects of defensive compounds and/or products released from these compounds on insect growth depend strongly on the background variation in nutritive leaf traits (Slansky and Wheeler 1992). For example, compensatory feeding on low nutritive diets increases the intake of defensive compounds, and the availability of growth-limiting nutritive compounds may thus modify the effects of chemical defense (Simpson and Raubenheimer 2001; Haukioja 2003). In the present study, we compared HC- and LC-accessions of the same species and can exclude other factors that may vary strongly among different plant species. Yet, we also quantified the most important nutritive and defensive traits of the food plants, i.e., soluble protein concentration and total phenolics, as well as leaf mass per area, which means an approximate measure for crude fiber content. None of these traits differed significantly among young leaves of the accessions (Table 1). Additional analyses indicate that there was no variation of the digestible sugar concentration in leaf developmental stages among the accessions studied (unpublished data). Differences observed among beetles reared on HC- and LC-accessions resulted from differences in plants cyanogenic features.

The quality of resources encountered during development can have effects on individual performance and on reproductive success (Janzen 1977; Fox et al. 1995; Ojeda-Avila et al. 2003). Reproductive success is even more relevant than growth rates, as it directly determines fitness. Females that had been reared as larvae and that were feeding as adults on HC-leaves produced significantly fewer eggs than females on LC-leaves (Fig. 2; Table 3). This effect intensified with every consecutive generation. It was also observed in different episodes of oviposition within a single generation. Females of the fourth generation that had developed consecutively on HC-plants produced smaller second clutches than comparable females that had developed on LC-plants or “Saxa” plants. Fourth generation females from HC-plants rarely produced a third egg clutch (Fig. 2). The size of the first clutches, in contrast, was affected.

The quality of eggs was affected by the type of food utilized by females. Eggs laid by females that fed on HC-leaves hatched at lower rates than those laid by females that fed on

Table 4 Indices of consumption, growth, and food utilization

Accession	Cyanogenic Status	Relative Growth rate (mg)	Relative Consumption Rate (mg)	Efficiency of Conversion of Ingested Food (mg)
2116	HC	0.26 ± 0.09^{b}	0.42 ± 0.07^{a}	63.20 ± 16.98^{b}
2357	HC	0.28 ± 0.07^{ab}	0.37 ± 0.09^{ab}	78.19 ± 26.30^{ab}
WT	HC	0.25 ± 0.04^{b}	0.34 ± 0.07^{ab}	76.24 ± 22.25^{ab}
8067	LC	0.42 ± 0.05^{c}	0.31 ± 0.05^{b}	142.33 ± 35.11^{c}
8079	LC	0.38 ± 0.13^{ac}	0.29 ± 0.07^{b}	136.04 ± 26.19^{c}
8071	LC	0.43 ± 0.06^{c}	0.29 ± 0.08^{b}	160.86 ± 53.76^{c}
Saxa	Non-Cyanogenic	0.39 ± 0.06^{c}	0.33 ± 0.08^{b}	123.14 ± 23.39^{ac}

Larvae were reared on leaves of different *P. lunatus* accessions. Values shown for different performance efficiency are means (±SD). Letters indicate significant differences calculated by a *post hoc* test (Tukey's HSD; $P<0.001$) after one-way ANOVA.

LC-leaves or "Saxa" leaves (Fig. 3). This effect appeared to be cumulative and became stronger with each successive generation. So far, such long-term effects of cyanogenesis have been underestimated in analyses of herbivore–plant interactions. However, our study suggests that the long-term effects of cyanogenic features on herbivores must be considered. Future studies should reassess the potential costs arising from host plant defensive traits even on specialist herbivores.

We did not carry out a comparative study between a specialist and a generalist. Thus, we cannot exclude in this study that a generalist herbivore would be affected more severely by cyanogenic features of lima beans. However, negative long-term effects of host plant cyanogenic features on the specialized Mexican bean beetle were definitive. Although negative long-term effects of host plant cyanogenesis were established, the exact physiological mechanism by which this effect is mediated is unclear. Cyanide may interfere with P450 cytochromes involved in final steps of cell respiratory pathways or it may affect nutrient availability for the insects by inhibition of digestive enzymatic activities (Solomonson 1988). As we did not create a balance between cyanide incorporated in bound form as cyanogenic precursors and cyanide released during the feeding process of beetles, we cannot tell in what way the insects came into contact with inhibitory concentrations of cyanide. However, previous studies with the generalist herbivore *Schistocerca gregaria* (Ballhorn et al. 2005) showed that even lima beans with high concentrations of cyanide-containing precursors and highly active β-glucosidase—and therefore rapid and substantial release of gaseous HCN as a reaction to insect feeding—released only about 5–20% of the HCN present as precursors in the consumed leaf material. Thus, as observed in this study, feeding on all high HCNp plants should result in substantial incorporation of cyanide in bound form. The incorporation of intact precursors and the subsequent release of HCN within the gastrointestinal tract by herbivore-internal β-glucosidases appear to be the most harmful way to contact plant cyanide (Ballhorn et al. 2005; Amelot et al. 2006).

In summary, our results demonstrate that the Mexican bean beetle, although being an oligophagous species, is still quantitatively affected by its host plant cyanogenic features. As variation of HCNc among plants that contain high amounts of cyanogenic precursors did not result in differences in food utilization, development, or reproduction, the HCNp is the crucial cyanogenic parameter that affects performance of Mexican bean beetles.

Acknowledgements We thank Dr. Christoph Reisdorff and Dr. Helmut Kassner for assistance and helpful comments and thank the "Institute of Plant Genetics and Crop Plant Research (IPK)" in Gatersleben, Germany, for providing seed material of *P. lunatus*.

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Keywords Toxic nectar · Secondary compounds · Terpenoids · Honeybees · Deterrence · Toxicity

Introduction

Apart from sugars and amino acids, secondary compounds such as phenolics, alkaloids, and terpenoids are of widespread occurrence in nectar (Baker and Baker 1982; Nicolson and Thornburg 2007). Thus far, studies of toxic nectars have focused on the possible fitness benefits to the plants that produce them (Adler 2000) rather than on the sensitivity of natural floral visitors to the nectars. Rhoades and Bergdahl (1981) argued that the ability of specific pollinators to tolerate toxic or repellent compounds would serve as a coevolutionary mechanism to exclude nectar thieves and encourage legitimate pollinators. Adler's (2000) review of toxic nectar emphasized that there have been, first, many studies on the chemistry of secondary compounds in nectar and, second, many demonstrations of nectar toxicity to honeybees (in which the responsible compounds are usually not identified). Less common, however, are studies in a third category: those that make the link between chemistry and toxicity by presenting nectar toxins in sucrose solutions to pollinators in the field (Stephenson 1982; Masters 1991; Hagler and Buchmann 1993; Singaravelan et al. 2005; Liu et al. 2007).

The efficient recruitment behavior of honeybees ensures that good nectar sources are exploited rapidly: colonies focus on the best nectar sources in a large area by working relatively few patches at any one time and making frequent adjustments to the number of foragers at those patches (Visscher and Seeley 1982). The response of bees to a particular nectar depends on the ecological context, especially other available nectar sources (Gegear et al. 2007). This is an important consideration for experiments that involve feeder choices in a natural environment. In a classic study, Lindauer (1948) showed that the threshold sucrose concentration for eliciting recruitment behavior in honeybees declined from ~55% w/w during the main nectar flow in spring to around 3.5% in mid-summer when bee forage became scarce and competition was intense.

During periods of dearth, colonies of honeybees have a considerably more restricted range of plant choices, and in the course of the seasons, they are inevitably forced by floral circumstances to readjust their threshold of nectar acceptability. It is in this context of natural dearth in southern China that honeybees, *Apis cerana*, forage on the toxic nectar of a perennial vine, *Tripterygium hypoglaucum* (Level.) Hutch (Celastraceae). Powdered roots of this plant, also known as Lei Gong Teng ('Thunder God Vine'), have been used in traditional Chinese medicine for 2,000 years, and the main active ingredient is a potent diterpenoid, triptolide (Zhen et al. 1995; Qiu and Kao 2003; Brinker and Raskin 2005; Brinker et al. 2007). In this paper, we report the results of experiments on the foraging sensitivity of honeybees to the honey of this plant and its toxicological effects, and assess whether the bees alter their nectar acceptability threshold under conditions of dearth.

Methods and Materials

The toxic honey of *T. hypoglaucum* was collected from five *A. cerana* colonies in Yaoan county, Yunnan Province, China in June 2006, when *T. hypoglaucum* was one of only very few plants providing nectar for foraging insects. Pollen grains from both *T. hypoglaucum* flowers and the honey derived from this plant species were examined microscopically to confirm their common origin.

Determination of Triptolide The concentration of triptolide in honey derived from *T. hypoglaucum* was measured by using high-performance liquid chromatography (HPLC), preceded by a solid-phase extraction step. A 25-g sample of toxic honey was placed in a centrifuge tube with 40 ml water, and when it was completely dissolved, 40 ml ethyl acetate and 30 ml petroleum ether were added to the honey solution. The sample was treated ultrasonically to accelerate dissolution and then centrifuged for 10 min at 3,000 rpm. Finally, the supernatant was transferred to another centrifuge tube and was re-extracted with 30 ml ethyl acetate and 20 ml petroleum ether. The supernatant was concentrated in a rotary evaporator, and the residue was redissolved in ethyl acetate and filtered with 20 ml ethyl acetate through a neutral aluminum column, which was immersed in ethyl acetate. The eluant was collected and concentrated to dryness, then dissolved in 1 ml methanol and filtered through a 0.45-μm millipore membrane.

For standard solutions, 2.5 mg of triptolide (Medical Research Institute, Fujian Province, China, Fig. 1) were dissolved in 5 ml methanol to obtain a 0.5 mg/ml solution and kept at 4°C. Chromatographic conditions were as follows: Symmetry® C18 column (4.6×150 mm, 5 μm), acetonitrile–water (30:70) as the mobile phase, flow rate of 1 ml/min, detection at a wavelength of 218 nm, and sample size of 20 μl. The same method was used to test for triptolide in the honey of *Vicia sativa* L. (Fabaceae) and *Elsholtzia ciliata* (Thunb.) Hyl. (Lamiaceae): three samples were tested for each species.

Cage Tests To establish toxicity of the *T. hypoglaucum* honey samples, six groups of 40 young adult worker bees were collected randomly from one beehive and placed into each of six cages. A test group of three cages were given toxic honey as food and a control group of three cages received normal honey as food. Honey and powdered sugar were mixed (1:1) to make the cake to feed the bees. Cages were kept in an incubator at 25°C and 70% relative humidity. The number of dead bees in each cage was counted three times a day for 6 d.

Feeder Experiments Honeybee foraging preferences for normal and triptolide honey were tested in a series of experiments. A feeder containing 30% w/w sucrose solution was placed on a small square of blue board, 15×15 cm, at the entrance of an *A. cerana* observation hive. When a few foraging workers had been lured to it and had begun to imbibe the solution, the feeder and bees were moved to a site 5 m away. After having consumed

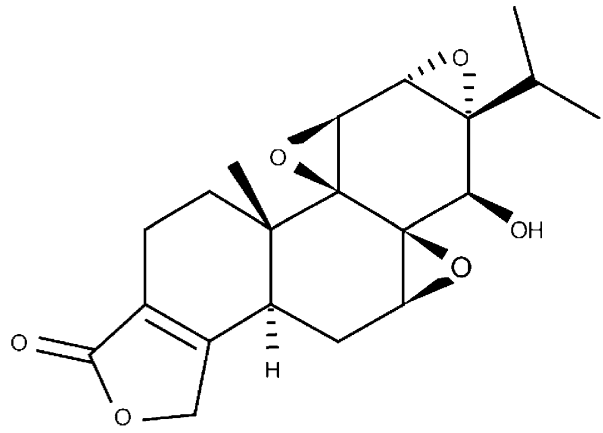


Fig. 1 Structure of triptolide

sufficient sucrose solution, these bees returned to their hive, and about 15 min later, additional, newly recruited foragers arrived at the feeder. At this point, we began to use paired feeders (A and B), which were placed at the same site. Visiting frequencies (the number of bees visiting in 10 min) and imbibition times of individual bees (sec) were recorded at both feeders. The ambient temperature was around 25°C during these tests.

Initially, both feeders (A and B) contained the same 30% sucrose solution. Then, the position of the feeders was reversed, and recordings continued for three more exchanges of position to assess whether there might be a position bias in the bees' responses. We then compared honeybee responses to the toxic honey of *T. hypoglaucum* and the nontoxic normal honeys of *V. sativa* and *E. ciliata* (all diluted 1:3 with water). We also compared the responses to 30% sucrose solution, with and without added triptolide at a concentration of 0.6 μg/g. This triptolide concentration is higher than in the diluted honey of *T. hypoglaucum*, but additional toxic compounds are likely to be present in the honey. All test solutions were of a similar sugar concentration, thus excluding effects of viscosity on imbibition time. Finally, we simulated the dearth conditions under which the bees actually forage on *T. hypoglaucum* by removing the normal honey and measuring responses to *T. hypoglaucum* honey alone.

Statistical Analysis Data are presented as mean±SD. Pearson χ^2 tests were used to test for significant differences in the percentage mortality of worker bees between the control and test groups. Log-linear *G* test analysis was used to test for homogeneity of mortality rates within the groups (Sokal and Rohlf 1995).

Analysis of variance (ANOVA) procedures with dependent samples (feeders A and B) were used to test for significant group effects in the visiting frequencies and imbibition times of foragers. Levene's test was used to test for homogeneity of the variances, and heterogeneity of the variances was stabilized after a square–root transformation of the visiting frequencies. Scheffé post-hoc multiple comparison tests were used for significant group effects. In one comparison, small sample size necessitated the use of a Mann–Whitney *U* test. All tests were performed by using Statistica© (StatSoft 2006).

Results

Determination of Triptolide Pollen grains from both *T. hypoglaucum* flowers and the honey derived from these flowers were examined, and it was confirmed microscopically that the honey had the same pollen grains as the flowers.

In the HPLC tests, 20, 10, 5, 2, and 1 μl of the authentic triptolide solution (0.5 mg/ml) were run to obtain the standard chromatogram for the pure compound. Using the peak area as the ordinate and concentration as the abscissa, a regression equation (y=2,511,038x+ 165,934, r=0.999) was obtained demonstrating linearity over the range of 1–20 μg/g. Repeated HPLC analyses of the authentic toxin established that the relative standard deviation (RSD) of the peak area was 2.63%. Samples (20 μl) of the toxic *T. hypoglaucum* honey were injected three times, with a 30-min run. The results showed that the retention time and the peak area were both stable; the RSD of the peak area was 0.61%. Triptolide was present in honey at a concentration of 0.61±0.11 μg/g (n=5) but was absent from the honeys of *V. sativa* and *E. ciliata*.

Cage Tests The toxicity of *T. hypoglaucum* honey was reflected in the numbers of dead bees in each cage. The results showed that significantly more worker bees in the test group (toxic honey) died within 6 d (68.3±25.0%) compared to the control group with nontoxic *E. ciliata* honey, where only 15.8±12.3% died (χ^2=67.9, *df*=1, *P*<0.001). The mortality rates were homogeneous among the cages in the control group (*G* test: G^2=4.5, *df*=2, *P*=0.107), while in the test group (*G* test: G^2=15.3, *df*=2, *P*<0.001), two cages were homogeneous, but in one cage a large number of bees (22) died on the sixth day.

Feeder Experiments When both feeders A and B contained 30% sucrose, Scheffé post-hoc results showed no significant differences in the visiting frequencies and imbibition times of foragers visiting the feeders (number of visits in 10 min: A=145.1±41.9, B=143.7±42.1, *N*=9 replicates; imbibition times: A=26.2±10.2 sec, B=27.8±13.6 sec, *N*=20 bees; Scheffé: *P*>0.05). This establishes that there was no position bias in the worker bees.

Subsequent tests measured selectivity in the foraging behavior of worker honeybees for the test groups (*T. hypoglaucum*, sucrose solution with triptolide) and control groups (*V. sativa* and *E. ciliata* honeys, sucrose solution only). ANOVA showed significant differences in the visiting frequencies and imbibition times of foragers between the control and test groups (number of visits: $F_{1,\ 24}$=72.3, *P*<0.001, Fig. 2; imbibition times: $F_{1,\ 115}$=43.0, *P*<0.001, Fig. 3) and significant group and food type interaction effects (number of visits: $F_{4,\ 24}$=13.1, *P*<0.001, Fig. 2; imbibition times: $F_{4,\ 115}$=6.6, *P*<0.001, Fig. 3).

Post-hoc test results showed that visiting frequencies to the feeders with normal honey were significantly higher than to those with toxic honey (*V. sativa*=275.3±75.6 bees per 10-min period, *T. hypoglaucum*=54.2±29.4, and *E. ciliata*=304.0±179.3, *T. hypoglaucum*=118.8±75.8, all *N*=6; Scheffé: *P*<0.001, Fig. 2). Likewise, the imbibition times of foragers at the feeders with normal honey were longer than at those with toxic honey (*V. sativa*=24.6±

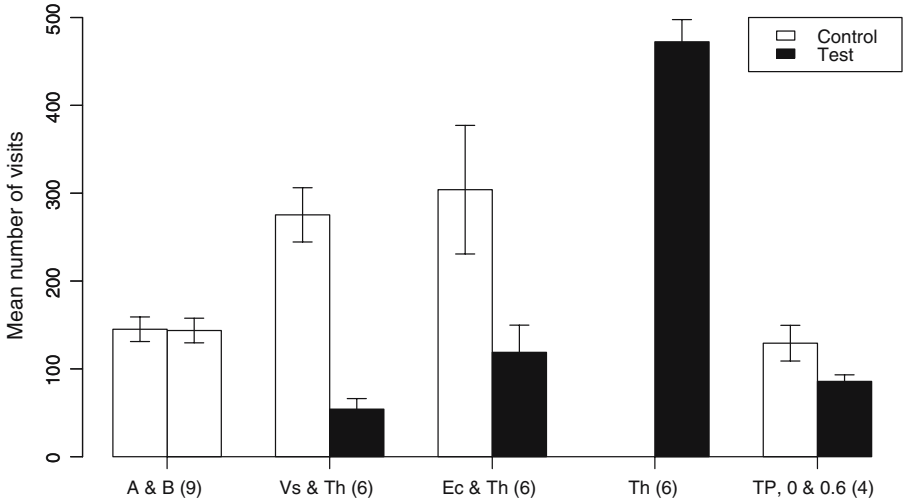


Fig. 2 Bee visits to feeders (10-min counts, means±SE, number of replicates in *parentheses*). In all cases, the control food is shown by *clear bars* and the toxic food by *black bars*. *A & B* Identical feeders containing 30% w/w sucrose solution, *Vs & Th* honeys of *Vicia sativa* and *Tripterygium hypoglaucum*, *Ec & Th* honeys of *Elsholtzia ciliata* and *Tripterygium hypoglaucum*, *Th* honey of *Tripterygium hypoglaucum.* All honeys were diluted 1:3 with water. *TP, 0 & 0.6* 30% sucrose solution and the same solution containing 0.6 μg/g triptolide

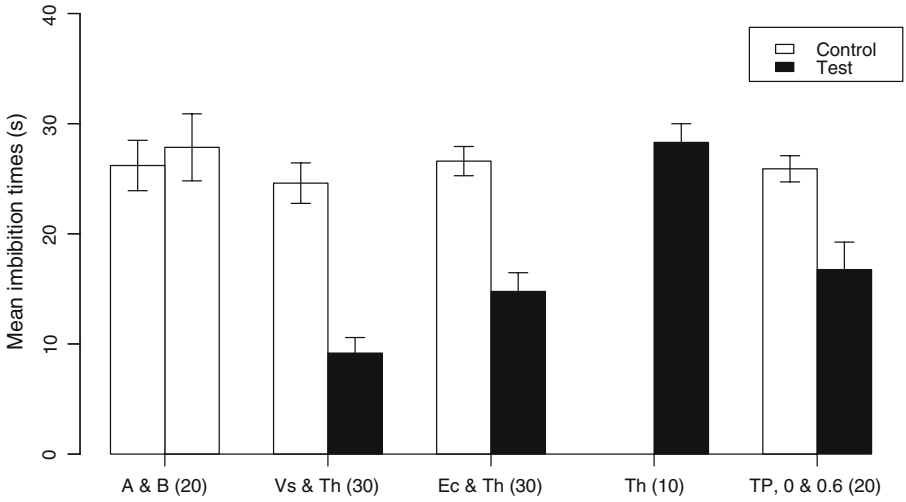


Fig. 3 Imbibition times (sec, means±SE, number of bees in *parentheses*). In all cases, the control food is shown by *clear bars* and the toxic food by *black bars*. *A & B* Identical feeders containing 30% w/w sucrose solution, *Vs & Th* honeys of *Vicia sativa* and *Tripterygium hypoglaucum*, *Ec & Th* honeys of *Elsholtzia ciliata* and *Tripterygium hypoglaucum*, *Th* honey of *Tripterygium hypoglaucum.* All honeys were diluted 1:3 with water. *TP, 0 & 0.6* 30% sucrose solution and the same solution containing 0.6 μg/g triptolide

10.1 sec, *T. hypoglaucum*=9.2±7.7 sec, and *E. ciliata*: 26.6±7.3 sec, *T. hypoglaucum*=14.8±9.3 sec, all N=30; Scheffé: P<0.001, Fig. 3).

There were significant differences in the visiting frequencies of foragers to feeders that contained sucrose solution with and without triptolide (normal=129.2±40.6, triptolide=85.7±14.9, N=4; Mann–Whitney: U=1.0, P=0.043). There was likewise a significant difference in imbibition times (normal=25.9±5.3 sec, triptolide=16.7±11.1 sec, N=20; Scheffé: P=0.050).

When the feeder of normal honey of *E. ciliata* was removed from the feeding site, leaving only the feeder of toxic *T. hypoglaucum* honey, within a few seconds, all of the foraging bees landed and began to imbibe the toxic honey. The visiting frequency and imbibition period increased significantly, reaching and even exceeding levels previously recorded at the feeder of normal honey (ANOVA: visits [N=6]=472.3±61.9, $F_{2,\ 15}$=13.5, P<0.001; imbibition times [N=10]=28.3±5.3 sec, $F_{2,\ 47}$=16.3, P<0.001). Post-hoc test results showed a significant increase in mean visiting frequencies and imbibition times of foragers at the feeders with toxic honey after *E. ciliata* honey was removed (visits, Scheffé: P<0.001; imbibition times, Scheffé: P<0.001) but no significant difference in mean visiting frequencies and imbibition times between toxic honey after *E. ciliata* was removed and normal honey when toxic honey was also present (visits, Scheffé: P=0.077; imbibition times, Scheffé: P=0.923).

Discussion

Terpenoids, especially monoterpenoids such as linalool, are common constituents of floral scent (Raguso and Pichersky 1999), and it is usually assumed that their presence in nectar is due to passive acquisition from scented corollas (Raguso 2004). Kessler and Baldwin (2006) recently reported a large number of secondary compounds in the headspace, nectar,

and floral parts of *Nicotiana attenuata* (Solanaceae), including many terpenoids. When defensive compounds are produced in other plant tissues and appear in phloem, it is likely that they will also occur in nectar (Adler 2000). Triptolide is presumably present in nectar as a consequence of its occurrence in the leaves and roots of *T. hypoglaucum*: the roots are used for commercial extraction of this compound (Brinker and Raskin 2005). It was suggested by Detzel and Wink (1993) that terpenoids may be less toxic or repellent to bees than other secondary compounds because of the role they play in the attraction of pollinators by floral volatiles. The olfactory responses of bees to terpenoids in floral volatiles are well known (Pham-Delegue et al. 1997; Laska et al. 1999). However, to our knowledge, the study by Detzel and Wink (1993) is the only previous test of the responses of bees to terpenoids in artificial nectar.

Although the triptolide concentration in nectar of *T. hypoglaucum* is unknown, we cannot assume that it is much lower than in honey because of the concentrating process. Phenolics in the nectar of *Aloe littoralis* appear to be deactivated during the manufacture of honey (Liu et al. 2005), and nicotine concentrations in fresh honey have been recorded as 90% lower than the concentrations in experimental diets from which that honey was manufactured (Singaravelan et al. 2006). Almond honey also contains lower amygdalin concentrations than the nectar (London-Shafir et al., 2003). Naef et al. (2004) compared the biochemistry of nectar, honeybee crop contents, and ripe honey and attributed changes in the chemistry of terpenoids and other compounds to the highly oxidative atmosphere in the hive.

Visiting frequencies measured at feeders demonstrated a strong preference of the worker bees for normal sucrose solution or diluted honey as opposed to the toxic equivalent. Similarly, in all comparisons, the imbibition rates were significantly shorter on the toxic honey and sucrose solution when the bees had a choice between toxic and nontoxic diets. Because any honey is a complex mixture of many compounds, it was necessary to demonstrate that pure triptolide had the same effects as did the toxic honey. This suggests that the bees perceive differences between the diets and reduce the risks associated with a toxic honey by reducing the amount of uptake. This is supported by the fact that when there was no alternative to a toxic honey, the visiting frequencies and imbibition times increased to the same levels as for normal honey. Indeed, this is precisely what happens under dearth conditions when there is little flower choice, and people in southern China avoid collecting honey during the flowering of *Tripterygium*. Although our cage and feeder experiments showed that toxic nectar of *T. hypoglaucum* has a significant toxicological effect on honeybees and acts as a deterrent, the seasonal context is important.

Similar results to ours were reported by London-Shafir et al. (2003) for almond flowers, whose nectar contains amygdalin, a cyanogenic glycoside. Preference tests with sugar solutions containing various concentrations of amygdalin showed avoidance of amygdalin in early summer. However, bees did not differentiate among the different amygdalin concentrations in mid-summer, when floral resources are scarce and the bees are less discriminating. This also meant that the authors had to use a lower sugar concentration for their experiments in mid-summer (15 compared to 25%) to avoid overcrowding at the feeders.

Secondary compounds that are repellent to bees at some concentrations can sometimes stimulate them to feed. Recently, Singaravelan et al. (2005) tested the responses of honeybees to four compounds found naturally in floral nectar: nicotine, anabasine, caffeine, and amygdalin. Except for anabasine, naturally occurring concentrations had no deterrent effect, and low concentrations of nicotine and caffeine elicited a significant feeding preference. These compounds were presented to the bees in 20% sucrose solutions. In

addition, feeding experiments with caged bees showed no deleterious effect of natural concentrations of nicotine (Singaravelan et al. 2006).

Most studies (including the present one) use a single sugar concentration for feeding experiments with bees. However, an important recent finding is that the response of *A. cerana* to phenolics in artificial nectar depends on the sugar concentration of the nectar (Liu et al. 2007). Phenolics had an inhibitory effect at low and high sugar concentrations but an attractant effect in the mid-range of 15–35% w/w. Therefore, the attractive or deterrent effect of a secondary compound in nectar depends not only on the concentration of that particular compound but also on the sugar concentration.

In a recent study of the complex nectar chemistry of *N. attenuata* and its effect on pollinators, Kessler and Baldwin (2006) found that visit frequency and visit time were inversely correlated for hawkmoths and hummingbirds presented with choices involving various secondary metabolites. These authors suggest that if nectar repellents decrease visit time and thus the amount of nectar removed, while at the same time increasing visit frequency, pollination can be achieved with smaller nectar rewards. This does not, however, apply to *T. hypoglaucum*, where the presence of triptolide decreases both visit time and visit frequency of honeybees. The ecological consequences for reproduction in this plant with toxic nectar are not clear. However, our feeder experiments show that the bees are only partially deterred by nectar of *T. hypoglaucum* when given the option of good-quality nectar, and in the absence of alternative food sources, the threshold for acceptability of toxic honey is rapidly reduced. The responses of honeybees to differences in nectar concentration and to the variety of chemicals present in nectar are complex and depend on the ecological context.

Acknowledgements Financial support was granted by the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Science and the Yunnan Agricultural University of China.

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over constitutive defense mechanisms that have higher metabolic costs (Agrawal and Karban 1999). Inducible defense includes the emission of volatile organic compounds (VOCs) after herbivore attack. Herbivore-induced VOCs may defend the plant directly by repelling other herbivore species (Landolt 1993; Bernasconi et al. 1998; Kessler and Baldwin 2001) or indirectly by attracting natural enemies of the herbivore (predators and parasitoids) that exert control over the feeding herbivore. The latter phenomenon, referred to as "indirect defense" has been observed in many tritrophic systems (Dicke 1999; Turlings and Wäckers 2004).

Herbivore-induced VOCs include an array of terpenes (mono-, sesqui-, and homoterpenes) that are the result of an increased release of constitutively emitted terpenes (Vuorinen et al. 2004) as well as terpene synthesis *de novo*. Among the most commonly induced are the oxygenated monoterpene linalool, the sesquiterpenes β-caryophyllene and α-farnesene, and the homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (Holopainen 2004). In addition, many alcohols, esters, and aldehydes referred to as green leaf volatiles (GLVs) are also emitted (Paré and Tumlinson 1999). The activation of genes behind the synthesis of inducible terpenes (Van Poecke et al. 2001; Arimura et al. 2000, 2004) and their systemic emission (Paré and Tumlison 1999) support the view that they are not passively released. In contrast, GLVs are thought to be released from damaged plant organelles, 1 or 2 sec after mechanical damage (Fall et al. 1999). However, there is evidence that some can also be emitted by undamaged tissue (Holopainen 2004). The differences found in ratios among GLVs by the feeding of two different insect species on the same plant species (Turlings and Wäckers 2004) and their ability to induce the emission of VOCs (Ruther and Kleier 2005; Yan and Wang 2006) as well as to activate defense genes (Arimura et al. 2001) in neighboring plants suggest that they have an active role in ecological interactions. Individual terpenes and GLVs are enough to attract natural enemies (Kessler and Baldwin 2001; Reddy et al. 2002; Ibrahim et al. 2005). However, under natural conditions where many plant species coexist, natural enemies may rely on ratios between different compounds (Bruce et al. 2005) that are the result of qualitative or quantitative changes from the constitutively emitted VOC profile (Dicke and Van Loon 2000).

Biogenic VOCs, including terpenes and oxygenated compounds, are also involved in chemical processes that occur in the low atmosphere (Fuentes et al. 2000; Atkinson and Arey 2003; Holzinger et al. 2005). In particular, terpenes are not only a source of ozone (O_3), but also a source of secondary organic aerosols when oxidized with common oxidants from the troposphere (e.g., OH, O_3, and NO_3). Terpenes and some oxygenated compounds have short atmospheric life times that can range from seconds to hours when they react with O_3 (Atkinson and Arey 2003). O_3 concentrations are expected to increase in future environments (IPCC 2007). Although the exposure of herbivore-induced VOCs to O_3 results in the degradation of many terpenes and GLVs and consequently in altered ratios among the compounds, the orientation of two selected natural enemies is not disrupted (Pinto et al. 2007a). In the tritrophic system *Brassica oleracea–Plutella xylostella–Cotesia plutellae*, atmospherically stable herbivore-induced compounds such as benzyl cyanide (Scascighini et al. 2005) seem to be sufficient synomones for the parasitoids in O_3-enriched environments. They might act as long-distance cues under natural conditions, in which the presence of O_3 and other oxidants is unavoidable (Pinto et al. 2007a). However, the role of terpenes and GLVs as synomones for wasps that parasitize *Brassica* spp. herbivores is unclear.

Terpenes have been acknowledged as important synomones for predators and parasitoids (Dicke 1994), but they might not contribute to the attraction of parasitoids of *Brassica* spp. herbivores. The quantitative emission of terpenes released by herbivore-damaged plants does not differ greatly from those released by mechanically damaged or intact cabbage plants. Therefore, the relative proportion of terpenes in the herbivore-induced blend is low,

as other compounds are also induced (Mattiaci et al. 1994). Similarly, in the related species *B. oleracea* var. *gemmifera*, the amounts of terpenes induced by herbivory do not increase significantly over time suggesting that they do not correlate with preference of the parasitoid *Cotesia glomerata* (Scascighini et al. 2005). This observation suggests that herbivore-induced terpenoids might not play a crucial role in the orientation of parasitoids of *Brassica* spp. herbivores. GLVs are enhanced in *Brassica* spp. by herbivory (Mattiacci et al. 1994; Geervliet et al. 1997; Scascighini et al. 2005), but *C. plutellae* prefers herbivore-damaged over mechanically damaged plants (Shiojiri et al. 2000, 2006), which also emit large amounts of GLVs (Van Poecke et al. 2001 and references therein). This suggests that GLVs are not sufficient cues for this specialist parasitoid. GLVs emitted by cabbage plants can be also recognized by other predators (Reddy et al. 2002), and thus, other compounds may be more reliable cues for specialist parasitoids.

The oxidation of VOCs results in a series of more atmospherically stable compounds (Calogirou et al. 1999; Yu et al. 1999), and there is a likelihood that natural enemies may have learned to use these oxidation products as synomones. In the present study, we investigated the role of terpenes and GLVs and their oxidation products on the orientation of *C. plutellae* toward *P. xylostella*-damaged plants. By degrading herbivore-induced terpenes and GLVs with O_3, we tested whether the wasps show preference toward unoxidized herbivore-induced volatiles (herbivore-damaged plants at 0 nl l^{-1} O_3) or oxidized herbivore-induced volatiles (herbivore-damaged plants at 120 nl l^{-1} O_3).

Materials and Methods

Living Material and Induction of VOCs White cabbage (*B. oleracea* L. ssp. *capitata* L. cv. Lennox) was grown individually in polypropylene plastic pots filled with *Sphagnum* peat and sand (3:1 v/v) under greenhouse conditions. They were used for trials when they had developed four leaves (approximately 3 to 4 weeks). *P. xylostella* L. (Lepidoptera: Yponomeutidae) and its parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae) were reared on broccoli plants (*B. oleracea* L. ssp. *italica* Plenck) as described by Vuorinen et al. (2004). For inducing damage, ten second-instar *P. xylostella* were transferred onto the leaves of each plant, 48 hr before the bioassays, and removed just before the bioassays.

Setup for Oxidation of VOCs The setup is shown in Fig. 1. It is a modification of that previously described by Pinto et al. (2007a). The modification was aimed to compare the responses of the parasitoid between odor sources in either 0 or 120 nl l^{-1} O_3. A main 1.6 l min^{-1} airflow, previously passed through a charcoal filter, was equally divided in two (0.8 l min^{-1}). For the odor source exposed to 0 nl l^{-1} O_3, 0.8 l min^{-1} was directed into the vessel (22.3-l glass desiccators, Schott Duran, Schott AG, Mainz, Germany) that contained the odor source. The outgoing airflow from the vessel was divided in two, and 0.4 l min^{-1} was used for collecting VOCs or conducting behavioral tests. The remaining 0.4 l min^{-1} was removed via a vacuum pump. For the odor source destined for O_3 exposure, the remaining 0.8 l min^{-1} was directed through an O_3 generator (Model 1008-RS, Dasibi Environmental, California, USA) before contact with the plants. In addition to using the outgoing airflow from the vessel for VOC collections or behavioral tests in the conditions previously described, 0.4 l min^{-1} was diluted in an airflow and directed to an O_3 analyzer (Model O342M, Environment S.A., Poissy, France) to monitor the O_3 concentration targeted to 0 and 120 nl l^{-1}. O_3 scrubbers (potassium iodide—KI) were placed before the end of the line that was used for behavioral tests and VOC collections. The purpose of the scrubber was to remove O_3 before the Tenax TA and to avoid any O_3

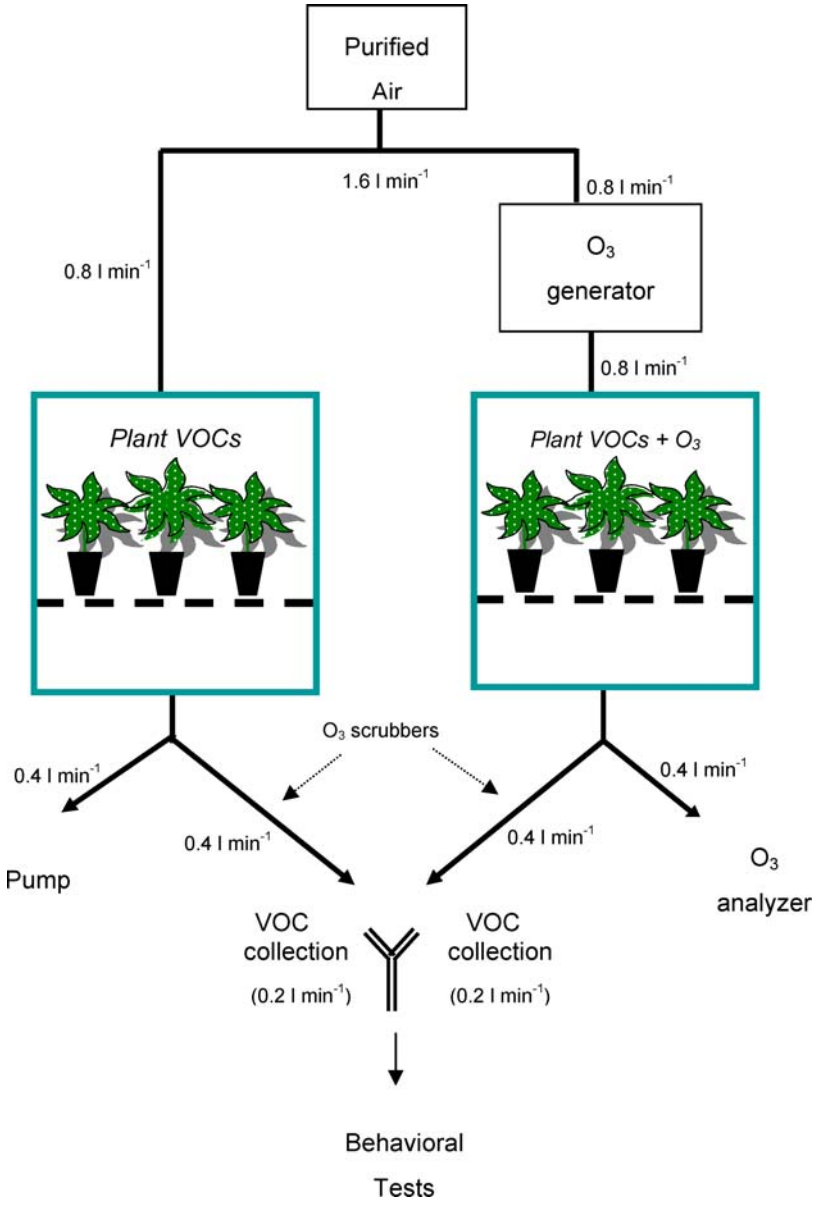


Fig. 1 Experimental setup for the VOC collection and behavioral tests. A main charcoal-filtered 1.6-l min^{-1} airflow was equally divided. 0.8 l min^{-1} were directed into one of the odor sources, whereas the remaining 0.8 l min^{-1} was previously passed through an O_3 generator targeted to 0 and 120 nl l^{-1} O_3 before being directed into one odor source. Out-flowing air from the odor sources was used for behavioral tests at 0.4 l min^{-1} or VOC collection at 0.2 l min^{-1}. The O_3 concentration was constantly measured

effect on the behavior of the parasitoid. To avoid any effect of the O_3 scrubber itself, a similar one was placed in both sides. The whole system was connected by Teflon tubing, and each odor source (experimental unit) consisted of a set of four intact or *P. xylostella*-damaged cabbage plants.

VOC Collection and Analyses VOC collection and analysis were previously described by Pinto et al. (2007a, b). In brief, VOCs were collected from each odor source (intact or damaged plants) onto approximately 150 mg Tenax TA adsorbent (mesh 60/80, Supelco, Pennsylvania, USA) by suction with a vacuum pump at a rate of 0.2 ml min^{-1}. Four replicates were collected for each herbivore treatment. VOCs were analyzed by gas chromatography–mass spectrometry (GC-MS, Hewlett Packard GC type 6890, MSD 5973). Trapped compounds were thermodesorbed (ATD400 Automatic Thermal Desorption System) at 250°C for 10 min, cryofocused at −30°C and injected onto an HP-5MS capillary column (50 m long, 0.20 mm inner diameter, 0.33 mm film thickness, Hewlett Packard). Compounds were identified with pure standards and by comparing their mass spectra to those in the Wiley Library. Only α-thujene, (*E,E*)-α-farnesene, and benzyl cyanide, which were not present in the pure standard, were quantified on the basis of α-pinene, (*E,E*)-β-farnesene, and the internal standard 1-chlor-octane, respectively, also present in the pure standard. Emissions in filtered air were calculated as ng g^{-1} dry weight hr^{-1}, and concentrations at the different O_3 treatments were expressed in ng l^{-1}.

Behavioral Tests For the experiment, 3–4-d-old female *Cotesia* wasps were used. Before the experiment, wasps were offered a *P. xylostella*-infested plant (infested as previously described) for 20 min to increase responsiveness (Potting et al. 1999). The behavioral tests were previously described by Pinto et al. (2007a). In brief, wasps were introduced individually to the end of a Y-tube (diameter=1.6 cm, main arm, 10.5 cm, other arms 10 cm, and angle between them 90°). They were left to choose between the two odor sources for a maximum of 5 min. A "choice" was recorded when they walked further than two thirds of one arm and remained in that area for at least 5 sec. Otherwise, a "no choice" was registered. We assessed the preference of *C. plutellae* between the VOCs emitted by intact (control) plants at 0 nl l^{-1} O_3 and *P. xylostella*-damaged plants 120 nl l^{-1} O_3 and between VOCs emitted by *P. xylostella*-damaged plants at 0 and 120 nl l^{-1} O_3. As a control, we conducted the same bioassays with both odor sources in the absence of O_3. Six and five pairs of odor sources were used for the intact vs. *P. xylostella*-damaged plants bioassays and the *P. xylostella*-damaged vs. *P. xylostella*-damaged plants bioassays, respectively, and no more than 15 insects were tested per each pair of odor sources.

Statistical Analyses Statistical analyses were performed by using the SPSS 14.0 for Windows Statistical Package. Before the analysis, normality was tested, and some compounds were transformed by calculating the inverse ($1/x$). Differences between the emission rates from intact and herbivore-damaged plants in the absence of O_3 were analyzed by using the independent-samples *t* test. The effect of the O_3 treatment on each individual compound from the herbivore-induced VOC blend was analyzed with the paired-samples *t* test. Only (*Z*)-3-hexen-1-ol was not normally distributed and was analyzed using the nonparametric Wilcoxon Signed Rank test. Additionally, the concentration of the oxidized herbivore-induced VOCs was compared with that of VOCs from intact plants with the independent-samples *t* test, with the exception of (*Z*)-3-hexen-1-ol that was analyzed with the nonparametric Mann–Whitney *U* test. The behavioral responses of the natural enemies were analyzed with the nonparametric Binomial test.

Results

VOCs in the Headspace of Cabbage Plants The monoterpenes α-thujene, α-pinene, sabinene, β-myrcene, D-limonene, and 1,8-cineole were emitted by intact plants. *P. xylostella* induced an increase in their emissions but only D-limonene (P=0.023) was significantly increased. In addition, *P. xylostella* induced the emission of the homoterpene DMNT, the GLVs (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate, and the nitrile, benzyl cyanide (Table 1). The sesquiterpene (*E,E*)-α-farnesene was detected only in two samples (Table 1).

At 120 nl l^{-1} O_3, with exception of 1,8-cineole that increased (P=0.005), the concentration of all the compounds decreased. A statistically significant decrease was found for α-thujene (P=0.010), α-pinene (P=0.011), β-myrcene (P=0.008), D-limonene (P=0.007), and DMNT (P=0.007). (*E,E*)-α-Farnesene was not detected in any of the samples at 120 nl l^{-1} O_3 (Fig. 2). The concentration of (*Z*)-3-hexenyl-acetate was unable to be quantified at 120 nl l^{-1} O_3 because this compound was not separated from octanal by the column, and the latter is not affected by O_3.

When the concentration of the oxidized herbivore-induced VOCs was compared with that of VOCs from intact plants, the concentration of the constitutively emitted terpenes sabinene (P=0.048), D-limonene (P=0.529), and 1,8-cineole (P=0.001) were still higher after oxidation of herbivore-induced VOCs. Because herbivore-induced compounds were not emitted by intact plants, DMNT (P=0.009) and benzyl cyanide (P=0.007) were higher.

Behavioral Responses of C. plutellae *C. plutellae* clearly preferred the VOCs emitted by *P. xylostella*-damaged plants at 0 (P<0.001) and at 120 nl l^{-1} of O_3 (P=0.006) to intact plants at 0 nl l^{-1}. When *C. plutellae* was challenged to choose between two similar VOC blends of *P. xylostella*-damaged plants at 0 nl l^{-1} O_3, no preferences were observed. However, they preferred the induced VOC blend at 0 nl l^{-1} to that at 120 nl l^{-1} (P=0.001; Fig. 3).

Table 1 VOC emissions (ng g^{-1} DW hr^{-1}) from intact and *P. xylostella* (Px)-damaged cabbage in the ozone-free environment

	Intact	Px-damaged	*P* Value
α-Thujene[a]	1.6±0.2	2.0±0.3	0.304
α-Pinene	1.1±0.2	1.2±0.1	0.536
Sabinene	4.3±0.5	6.4±1.5	0.257
β-Myrcene	1.1±0.3	1.7±0.1	0.070
D-Limonene	7.5±1.1	11.7±0.8	*0.023*
1,8-Cineole	2.9±0.5	3.4±0.3	0.242
DMNT	0	10.5±1.2	*0.000*
(*E,E*)-α-Farnesene[a]	0	0.7±0.4	0.197
(*Z*)-3-Hexen-1-ol	0	2.5±0.9	0.060
(*Z*)-3-Hexenyl acetate	0	8.4±3.8	0.072
Benzyl cyanide[a]	0	1.2±0.2	*0.008*

Data are expressed as means±SE (N=4). *P* values from the independent-samples *t* test. Significant *P* values are shown in italics.

[a] Compound tentatively identified with the Wiley Library.

Discussion

In future environments, where O_3 is forecasted to prevail (IPCC 2007), parasitoids will not be able to choose spatially between two different O_3 environments, although in nature, the range of 1 hr mean daily maximum concentration may change from approximately 30 to approximately 90 nl l^{-1} within 2 d (Holopainen et al. 1994). However, we used this unrealistic situation to study the role of herbivore-induced terpenes and GLVs and their products after reacting with O_3 in the orientation of the parasitoid *C. plutellae* during host search. The identification of products resulting from the oxidation of terpenes is not easily achieved from direct GC-MS (Yu et al. 1998), and this approach may help to assess whether these products play an ecological role in interactions between plants and higher trophic levels. The results of a classical dual-choice bioassay under filtered air conditions support previous observations that *C. plutellae* prefers the VOCs emitted by *P. xylostella*-damaged plants over intact plants (Potting et al. 1999; Vuorinen et al. 2004). Elevated O_3 results in the degradation of induced terpenes and GLVs and does not affect orientation (Pinto et al. 2007a). This supports the view that *C. plutellae* is able to rely on compounds other than reactive terpenes and GLVs, such as benzyl cyanide (Scascighini et al. 2005) that are not significantly affected by O_3 exposure (Pinto et al. 2007a). However, it is possible that in the present study terpenes and GLVs, which were not totally degraded, also contributed to the attractiveness of the VOC blend. When the concentration of the oxidized herbivore-induced VOCs was compared with that of VOCs from intact plants, sabinene, 1,8-cineole, and DMNT, in addition to benzyl cyanide, which was not significantly degraded, were still present in significantly higher concentrations. Moreover, other inducible compounds that were not detected by the GC-MS after ozone exposure might have played a role in orientation of the wasps because the antennal receptors of the wasps are able to detect very

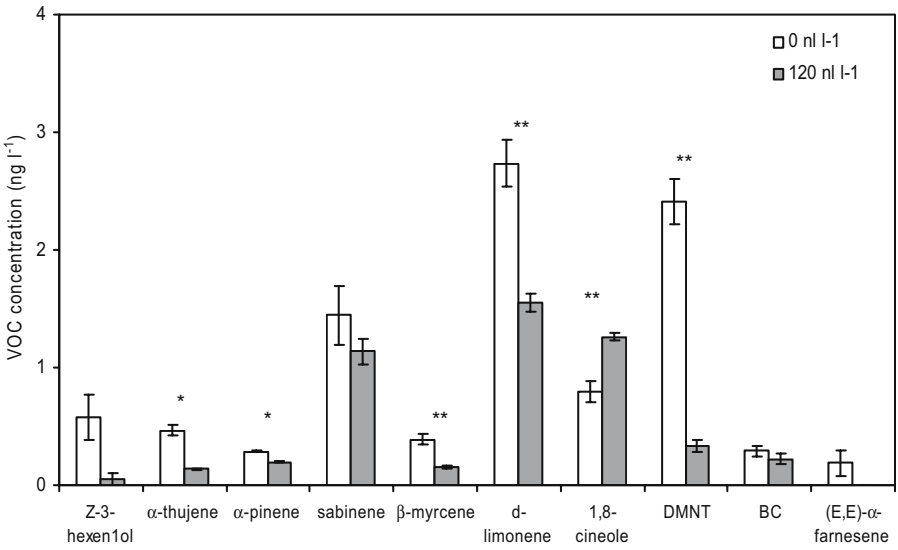


Fig. 2 VOCs (ng l^{-1}) in the headspace of four cabbage plants damaged by *P. xylostella* at 0 (*white bars*) and 120 nl l^{-1} (*gray bars*) of O_3. Data are expressed as means±SE (N=4). The *asterisks* show significance by the paired-samples *t* test (*asterisk*, $P<0.05$, *double asterisk*, $P<0.01$). *BC* Benzyl cyanide

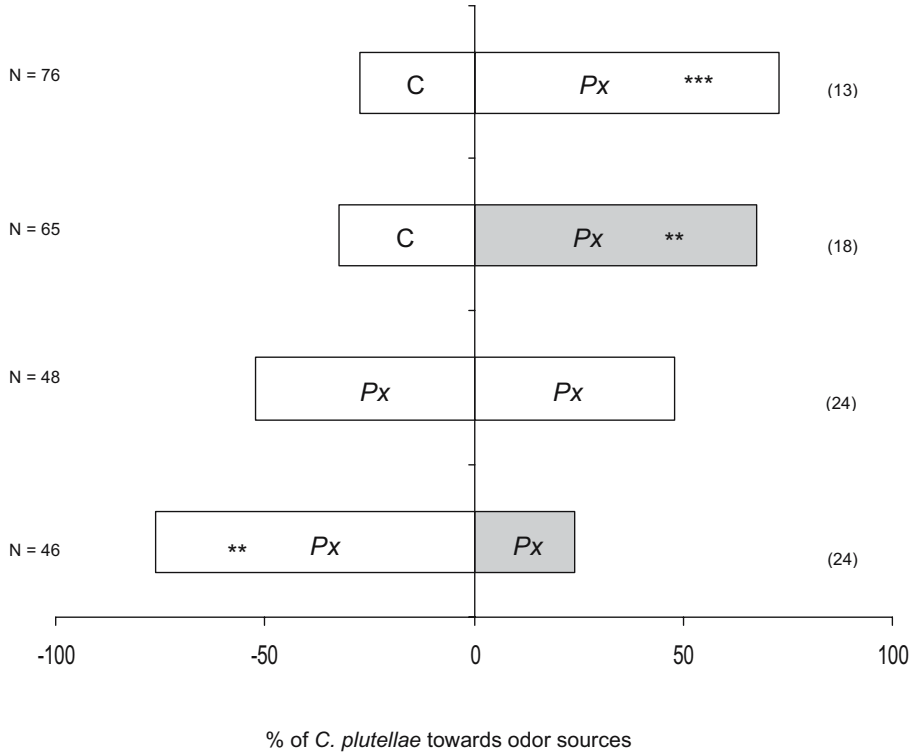


Fig. 3 The behavioral responses of the solitary endoparasitoid *C. plutellae*. The figure shows the percentage of female wasps choosing between control (*C*) and *P. xylostella*-damaged (*Px*) plants or between *P. xylostella*-damaged plants at 0 (*white bars*) or 120 nl l^{-1} (*gray bars*) of O_3-enriched airflows. The significances of the Binomial tests are shown with *asterisks* (*double asterisk*, $P<0.01$, *triple asterisk*, $P<0.001$). The total number of individuals making a choice is shown as *N*, and the number of wasps that did not choose between the two odor sources is in *parentheses* for each series

small volatile concentrations (Schoonhoven et al. 2006). On the other hand, if wasps relied on small concentrations of terpenes and GLVs, our observations would indicate that changes in the ratio among these compounds does not play a crucial role in the orientation of this species of wasp. At longer O_3 exposures, α-thujene, β-myrcene, and DMNT and (*Z*)-3-hexen-1-ol were not detected by the GC-MS at 120 nl l^{-1} (Pinto et al. 2007a). It is known that the extension of the reaction time can increase the amount of terpenes that react (Pommer et al. 2004).

When *C. plutellae* was challenged to choose between the unoxidized herbivore-induced VOCs and the oxidized herbivore-induced VOCs, a preference toward the unoxidized blend was seen. These results indicate that ozone might reduce their preference to cabbage plants. In addition, the results suggest that terpenes and GLVs increase the attractiveness of the herbivore-induced VOC blend. It is more likely that herbivore-induced compounds evoke a response from wasps, as constitutively emitted VOCs may not offer information on the presence of the host. DMNT is a terpene that is induced by the feeding of *P. xylostella*. This compound is emitted by many plant species exclusively after herbivore feeding, and its role as a synomone has been acknowledged (Karban and Baldwin 1997, for a review). In our

study, DMNT was emitted in great quantities but also degraded by O_3. It has been shown that plants infested with *P. xylostella*+*Pieris rapae* together emit lower amounts of DMNT than plants infested by *P. xylostella* alone that were more attractive to *C. plutellae* (Shiojiri et al. 2001). The authors of that study suggested that the behavioral response of *C. plutellae* was partly due to differences in the emission of DMNT, and this may have also occurred in our experiment. GLVs are known to attract *C. plutellae* (Reddy et al. 2002), and greater concentrations of the unoxidized VOC blend might also have contributed to the decision making of the wasps. *C. plutellae* has also been shown to be attracted to D-limonene itself, and this may be a behaviorally active compound in the VOC blend of cabbage plants (Ibrahim et al. 2005). Indeed, D-limonene is detected by the olfactory receptors of related *Cotesia* species (Smid et al. 2002). In this study, D-limonene was the only compound that showed a significant increase as a result of herbivore feeding. Therefore, it is possible that differences in its concentrations in the odor sources may contribute to wasp orientation. Herbivore-damaged *Brassica* species emit nitriles, isothiocyanates, and sulfides (Agelopoulos and Keller 1994; Mattiacci et al. 1994; Geervliet et al. 1997) that may be also reliable synomones for foraging parasitoids of *Brassica* herbivores (Agelopoulos and Keller 1994). We detected another nitrile, allyl nitrile (3-butene nitrile), and methyl thiocyanate in the herbivore-induced VOC blend. However, in contrast to benzyl cyanide, traces of these compounds were also detected in the emissions of intact plants. To our knowledge, the lifetimes of allyl nitrile and methyl thiocyanate when reacting with O_3 have not been reported. Although pure compounds should be used to assess accurately their lifespan in the atmosphere, we estimated their concentrations (data not shown) and assessed whether they significantly decreased after reacting with O_3. No differences were found for allyl nitrile (P=0.167, t test for paired samples) or methyl thiocyanate (P=0.198, t test for paired samples), which suggests that they did not readily react with O_3. We expected them to be emitted by both the unoxidized and the oxidized herbivore-induced VOC blend. Thus, we can assume that the preference of *C. plutellae* for damaged plants in the absence of O_3 is due to greater concentrations of terpenes and GLVs.

Before bioassays, wasps were offered a *Plutella*-infested plant for oviposition experience and to increase their response (Potting et al. 1999). Because parasitoids are able to learn to recognize individual compounds in mixture, as well as complex blends themselves (Meiners et al. 2003), it is possible that the wasps learned the odors from the cabbage plants that were not exposed to high O_3 concentrations. Therefore, they might have preferred the odor of damaged plants in filtered air over that one of damaged plants in elevated O_3.

In this study, we have shown that terpenes and GLVs play a role in the herbivore-induced VOC blend emitted by cabbage plants because *C. plutellae* are more attracted to cabbage plants where these compounds have not been oxidized by O_3. However, decreases and changes in ratio among these compounds do not affect the orientation of *C. plutellae*, suggesting that they can rely on other more O_3-stable-induced compounds. In nature, not only O_3 but other oxidizing agents such as OH radicals present in the atmosphere (Atkinson and Arey 2003) might degrade terpenes and GLVs that are in proximity to the odor source. It is possible that terpenes and GLVs provide information to *C. plutellae* on the presence of its host *P. xylostella* in the short range, but long-range orientation may rely on more stable compounds such as breakdown products of glucosinolates, including benzyl cyanide.

Acknowledgments The authors thank Timo Oksanen for assistance in developing the setup used in the experiment. D.M.P. is supported by the Finnish Graduate School of Environmental Science and Technology (ENSTE), J.K.H. by ISONET, MRTN-CT-2003-504720, J.K.H., and A-M.N. by the Academy of Finland (ESGEMO programme, decision no. 105209).

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which often results in the attraction of natural enemies of the herbivores (Dicke et al. 1990; Turlings et al. 1990; Vet and Dicke 1992; Takabayashi and Dicke 1996; Powell et al. 1998; Dicke 1999; Vet 1999). The vast majority of studies have considered simple tritrophic systems that involve plant defense responses to attack by a single herbivore species. However, plants in both natural and cultivated ecosystems are frequently subjected to simultaneous infestation by more than one species (Futuyma and Gould 1979; Thompson 1998; Rodriguez-Saona et al. 2003). Detailed analyses of plant volatile emissions have shown that different herbivore species often induce different volatile profiles in the same host plant species, even when the herbivores are of the same type, e.g., different lepidopteran caterpillars or different aphid species (Du et al. 1996, 1998; De Moraes et al. 1998; Turlings et al. 1998; Dicke 1999; Yan and Wang 2006). This specificity is often reflected in the responses of parasitoids and predators to herbivore-induced volatiles: specialist species in particular responding to specific plant volatile blends, while generalist species appear to be less selective (Du et al. 1996; Powell et al. 1998; De Moraes et al. 1998; Geervliet et al. 1998; Shiojiri et al. 2000; De Boer et al. 2005; Takabayashi et al. 2006; Yan and Wang 2006). Potentially, responses by parasitoids to specific, host-induced volatile blends could be compromised when two or more different herbivores are feeding on the same plant (Takabayashi et al. 2006).

Recent studies have investigated parasitoid responses to herbivore-induced plant volatiles when Brussels sprouts or cabbage plants (*Brassica oleracea* L.) were simultaneously infested with two lepidopteran caterpillars (Shiojiri et al. 2000, 2001; Vos et al. 2001). The presence of *Pieris rapae* L. caterpillars hindered the response of *Cotesia vestalis* (Haliday) to cabbages infested with its host *Plutella xylostella* L, whereas the presence of *P. xylostella* caterpillars appeared to enhance the response of *Cotesia glomerata* (L.) to plants infested with its host *P. rapae* (Shiojiri et al. 2000). It is interesting to note that in a different study, *C. glomerata* did not make a significant choice between Brussels sprout plants infested with the nonhost *P. xylostella* and plants infested with both *P. xylostella* and *P. rapae* (Vos et al. 2001). However, in these studies both herbivores were leaf-chewers, which probably both induce the same metabolic pathway. The presence of phloem-feeding whiteflies, *Bemisia argentifolii* Bellows and Perring, reduced by 60% the volatile emissions, from cotton plants infested with *Spodoptera exigua* Hübner caterpillars (Rodriguez-Saona et al. 2003). Fungal plant pathogens also induce changes in volatile emissions, and infection of maize plants by the fungus *Setosphaeria turcica* (Leonard and Suggs) reduced by 48% the total amount of volatiles released by maize plants infested with caterpillars of *Spodoptera littoralis* (Boid.) (Rostás et al. 2006). However, the presence of the fungus did not interfere with the responses of two hymenopteran parasitoids of *S. littoralis* to host-infested plants (Rostás et al. 2006).

In the present study, we investigated the responses of an aphid parasitoid, *Diaeretiella rapae* (McIntosh), to odors from cabbage plants infested with the peach-potato aphid *Myzus persicae* (Sulzer), in the presence and absence of a lepidopteran caterpillar, *P. xylostella*, to see whether presence of a chewing herbivore hindered the responses of a parasitoid to a phloem-feeding host. Brassicaceous plants are frequently infested by more than one herbivore species, including aphids and lepidopteran caterpillars, which often co-occur on the same leaf (Vos et al. 2001).

Materials and Methods

D. rapae was reared on *M. persicae* feeding on Chinese Cabbage plants, *Brassica chinensis* variety *pekinensis* (Rubr.) Sun., maintained in a Perspex cage in a controlled environment

room at 18±1°C, 75±5% relative humidity (RH), and at 18:6-hr light/dark photoperiod. The original laboratory culture had been set up in 1990 from mummies collected in the field from an oilseed rape, *Brassica napus* L., crop at Rothamsted Research farm in Hertfordshire, UK. *M. persicae* aphids were obtained from a laboratory culture maintained under the same conditions but in a separate room. Larvae of the diamondback moth *P. xylostella* were obtained from a laboratory culture maintained on oilseed rape plants in another controlled environment room at 22±1°C, 75±5% RH and at 18:6-hr light/dark photoperiod.

All experiments were done by using a Y-tube olfactometer made from 1-cm-internal diameter glass tubing with 5-cm-long arms, set at an angle of 45°, and with a 7-cm-long basal stem. The airflow through each of the olfactometer arms was created by a small electric pump and was maintained at 900 ml/min by means of a flow meter attached to each arm. The air flow to each arm was passed through activated charcoal to remove any contaminating odors, through a water trap to humidify the air, and then through a 5-l glass vessel containing the odor source before entering each olfactometer arm. The apparatus was lit by an overhead fluorescent light that emitted daylight wavelengths and was fitted with a plane-polarizing prismatic refractive cover to provide even illumination.

Female *D. rapae*, 24–48-hr-old, were tested in the following olfactometer bioassays:

1. Uninfested cabbage plant vs clean air
2. Cabbage plant infested with *M. persicae* vs clean air
3. Cabbage plant infested with *M. persicae* vs uninfested cabbage plant
4. Cabbage plant infested with *P. xylostella* vs uninfested cabbage plant
5. Cabbage plant infested with *M. persicae* vs cabbage plant infested with *P. xylostella*
6. Cabbage plant infested with *M. persicae* vs cabbage plant infested with both *M. persicae* and *P. xylostella*

For each of the six bioassays, 40 inexperienced followed by 40 experienced female parasitoids were tested. Inexperienced females had had no contact with either plant material or aphid hosts before testing, whereas experienced females had been allowed to oviposit in *M. persicae*. During the bioassays, a response was recorded if the parasitoid spent more than 15 sec beyond a line marked midway along an olfactometer arm, without crossing back out again. Each individual parasitoid was tested only once, and the positions of the two treatment arms of the olfactometer were reversed after each ten individuals to allow for any directional bias within the bioassay room. Young, potted cabbage plants (*B. oleracea* L.) with four to five leaves were used in all bioassays. To produce infested plants, either 100 *M. persicae* (mixed ages) or 50 first instars of *P. xylostella* were placed on the plants 2–3 d before use in the bioassays. For both inexperienced and experienced females, a χ^2 test was used to determine differences between numbers of parasitoids choosing different odor treatments within each bioassay.

Results

There were no significant differences between naïve and experienced *D. rapae* in their responses to plant volatiles in any of the bioassays. Therefore, the data were combined for further analyses and presentation (Fig. 1). Parasitoids showed a strong response ($P<0.001$) to odors from both uninfested and host aphid-infested plants when presented alongside clean air in the olfactometer (Fig. 1). However, when they were given a direct choice between odors from uninfested and aphid-infested plants, they made a significant ($P< 0.001$) choice

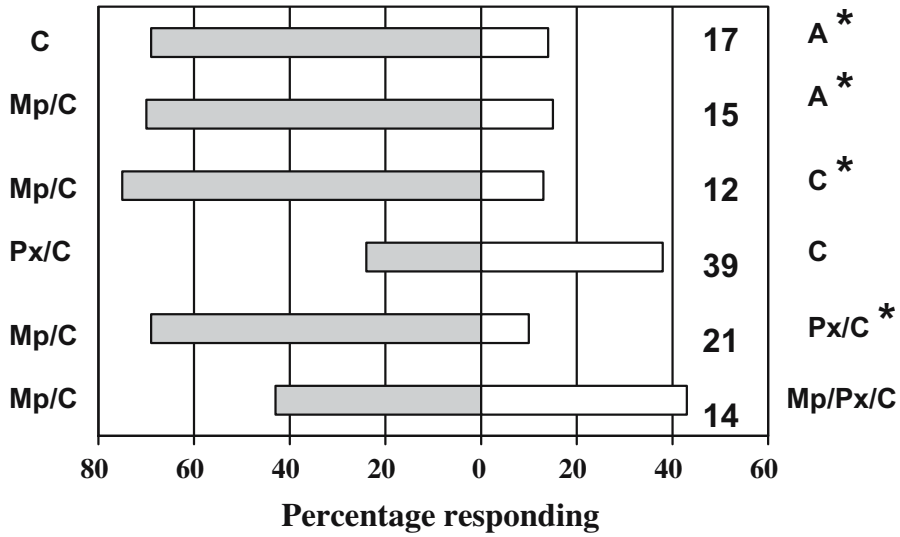


Fig. 1 Responses of female *Diaeretiella rapae* in a Y-tube olfactometer to odors from clean cabbage plants and plants infested with its aphid host, *Myzus persicae* and/or larvae of *Plutella xylostella*. N=80 for each test. *Numbers to the right of the bars* indicate percent nonresponders. *Asterisk*, $P<0.001$. (*A* clean air, *C* uninfested cabbage, *Mp/C* cabbage infested with *Myzus persicae*, *Px/C* cabbage infested with *Plutella xylostella*, *Mp/Px/C* cabbage infested with both *M. persicae* and *P. xylostella*)

of those from infested plants (Fig. 1). In contrast, they did not make a choice (P=0.058) between the odors of an uninfested plant and a plant infested with diamondback moth larvae (Fig. 1). When given a choice between odors from an aphid-infested plant and those from a plant infested with diamondback moth larvae, they significantly ($P<0.001$) chose the former (Fig. 1). Finally, the parasitoids responded equally to odors from a plant infested with aphids only and those from a plant infested with both aphids and moth larvae (Fig. 1).

Discussion

D. rapae females showed strong responses to volatiles from both uninfested and aphid-infested cabbage plants when compared with clean air, but when given a choice between these two odor sources, they strongly preferred that from aphid-infested plants. This indicates that the parasitoids respond to both noninduced brassicaceous plant volatiles and volatiles induced by host aphid feeding and that they can distinguish between the two. Girling et al. (2006) also recorded responses by *D. rapae* to *Arabidopsis thaliana* plants infested with *M. persicae* in Y-tube olfactometer bioassays, but in that study, only experienced parasitoids made a statistically significant choice between uninfested and aphid-infested plants. Similarly, experienced *D. rapae* females made a significant choice of *M. persicae*-infested turnip plants (*Brassica rapa* L.) over uninfested plants, whereas naïve females did not (Blande et al. 2007). Oviposition experience allows parasitoids to learn new olfactory foraging cues, but there is also evidence that it increases the level of existing responses (Turlings et al. 1993; Du et al. 1997; Guerrieri et al. 1997; Grasswitz 1998). It is possible that the host-induced volatile signals emitted by the *M. persicae*-infested cabbage plants used in our study were sufficiently strong to elicit a significant response from naïve parasitoid females, without a pretesting oviposition experience.

Previous studies have shown that feeding damage by *P. xylostella* larvae induces the release of volatiles from brassicaceous plants, including cabbage, and that parasitoids of the moth distinguish between uninfested and host-infested plants (Potting et al. 1999; Shiojiri et al. 2000, 2001; Bukovinsky et al. 2005; Ibrahim et al. 2005). Some parasitoids of other lepidopteran caterpillars that feed on Brassicaceae are also attracted to *P. xylostella*-damaged plants, although they do not use this species as a host (Agelopoulos and Keller 1994a; van Roosjen et al. 2003). However, the aphid parasitoid *D. rapae* did not distinguish between uninfested and *P. xylostella*-infested cabbage plants but did make a significant choice of *M. persicae*-infested plants over *P. xylostella*-infested plants. This supports the hypothesis that the aphid and the caterpillar induce different changes in the volatile profile of cabbage plants and that *D. rapae* females readily distinguish between the two. However, the aphid parasitoids did not distinguish between plants infested with aphids and plants infested with both the aphid and the caterpillar, indicating that the changes to the plant volatile profile induced by the caterpillar did not hinder the detection of aphid induced signals by *D. rapae*.

Brassicaceous plants release isothiocyanates, which are known to be used as foraging cues by both phytophagous and predatory insects, including *D. rapae* (Bartlet et al. 1997; Blight and Smart 1999; Bradburne and Mithen 2000; Cook et al. 2006; Blande et al. 2007). Feeding damage by *P. xylostella* larvae is known to induce the release of isothiocyanates from cabbage (Agelopoulos and Keller 1994b), so it might be expected that *D. rapae* would be attracted to *P. xylostella*-damaged plants. However, *P. xylostella* induces the release of allyl isothiocyanates (Agelopoulos and Keller 1994b), whereas *D. rapae* females are particularly attracted to 3-butenyl isothiocyanate (Bradburne and Mithen 2000; Blande et al. 2007).

The presence of a chewing insect that is known to induce changes in the volatile profile of cabbage plants did not disrupt the responses of a parasitoid to volatile cues induced by a sucking insect. This implies that the production of volatiles by cabbage plants in response to aphid feeding and that attract *D. rapae* is not hindered by concurrent caterpillar feeding damage. This makes sense because aphid-infested plants are frequently attacked simultaneously by chewing insects such as caterpillars, and the indirect defense strategy of plants is likely to have evolved in the context of multiple-herbivore attacks.

Acknowledgments The first author is indebted to the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK for financial support via the Underwood Fund and to The British Council for sponsoring the academic link with Rothamsted Research. Rothamsted Research receives grant-aided support from the BBSRC.

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implicated in CM host finding, attraction of the moth to host chemicals under field conditions is limited to a male-only response to *E*-β-farnesene (Coracini et al. 2004) and attraction of both sexes to ethyl-(*E*,*Z*)-2,4-decadienoate, the pear ester (Light et al. 2001).

Although a moth host kairomone might be expected to be used for the finding and selection of oviposition sites, it is unclear what role pear ester plays in codling moth location of oviposition sites. Pear ester is emitted from fully ripe pears (Jennings et al. 1964; Shiota 1990) late in the season after most CM oviposition has occurred. Females are attracted to this compound, even when unmated (Knight et al. 2002), before they would be ovipositing. Pear ester is more attractive to males than females (Thwaite et al. 2004), sometimes exclusively so (Kutinkova et al. 2005). In summary, our knowledge of CM attraction to pear ester is not always consistent with moth attraction to host odor to locate oviposition sites. Landolt and Guédot (2008) suggested an additional hypothesis that CM moth attraction to ripe fruit odor, and to pear ester specifically, may also be a food-finding behavior.

Many species of moths feed at sugar sources, such as floral and extrafloral plant nectars, fruit and fruit juices, saps and honeydews (Norris 1936). However, information on codling moth adult feeding is limited. They are attracted to sweet baits (Eyer 1931), they live longer and may lay more eggs when provided sugar (Howell 1981), and they can be trapped with acetic acid (Yothers 1927; Dethier 1947), which is a microbial fermentation product in sweet baits (Utrio and Eriksson 1977). Codling moths may also be attracted to ripe fruit odor as a food-finding behavior (Landolt and Guédot 2008). However, there are no published accounts of CM feeding on ripe or damaged ripe fruit in nature and no reports of acetic acid being emitted from ripe fruit (but see Chen et al. 2006 for acetic acid in extracts of ripe pear fruit).

We hypothesize that CM attraction to ripe fruit odorants such as pear ester (as a food-finding behavior) might be enhanced by the presence of acetic acid (as a sugar cue). Acetic acid has been found to be coattractive and synergistic with other compounds found in sweet baits for vespid wasps (Landolt 1998) and noctuid and pyralid moths (Landolt 2000). In this paper, we report laboratory and field experiments that test the hypothesis that acetic acid with pear ester acts synergistically to attract CM.

Methods and Materials

Laboratory Experiments

Insects All CM used in the laboratory experiments were reared by the Okanagan–Kootenay sterile insect-rearing facility in Osoyoos, British Columbia, Canada (Judd and Gardiner 2006). Larvae were reared on artificial diet (Brinton et al. 1969) at the Pacific Agri-Food Research Centre, Summerland, British Columbia where they were held at 24°C under a 16:8 h L/D photoperiod. Pupae were removed from the diet, sexed, and placed in separate and sealed 10-l plastic buckets. Newly emerged unmated adults were collected daily at the end of the scotophase and held under the same conditions for 48 h until tested. Mated female moths were obtained by placing both sexes together in a 2:1 male/female ratio into a single bucket on the first day of adult emergence. Responding females were dissected, and mated status was confirmed by the presence of spermatophores.

Flight tunnel Two flight tunnels described by Judd et al. (2005) were used to assess behavioral responses of naïve codling moths to attractants in clean air. Both tunnels were

used at the same time to expedite the timely completion of assays with moths provided from the colony. Unmated males were tested in the push-type tunnel, and females were tested in the pull-type tunnel (Judd et al. 2005). Mated females were tested in the pull-type tunnel because it has a removable cover that facilitated the removal of eggs laid on the tunnel walls. Testing of each sex in a different tunnel provided prevention of possible male responses to contamination from female pheromone. Details of moth handling and trapping procedures are reported by Judd et al. (2005). For each experiment, 48-h-old moths were placed in a lighted, 2°C chamber 60 min before scotophase and cooled for approximately 10 min. Immobilized moths were transferred to release cages (2 cm high by 9 cm diameter mesh cylinders attached to an 11-cm^2 base). Thirty minutes before the onset of the scotophase, caged moths were transferred to their respective flight tunnel and placed on a platform in the center of the tunnel within the overlapping plumes (visualized by prior smoke tests) of two lures when two were used. Lures were hung inside cylindrical polyester traps (13 cm long × 11 cm diameter) coated internally with Stickem Special™ (PheroTech, Delta, BC, Canada) to trap alighting moths. In all assays, the two traps were hung from a metal ring stand at the upwind end of each tunnel's flight section. Traps were hung in the same horizontal plane and separated by 20 cm from center to center. In the pull-type tunnel, moths were released 37 cm above the tunnel floor and 118 cm downwind from odor sources. In the push-type tunnel, moths were released 44 cm above the tunnel floor and 150 cm downwind from odor sources. At 1000 hours P.S.T. each day, flight-tunnel room lights were turned off, and both tunnels were lit by uniform, overhead white light provided by four 25 W incandescent bulbs (Philips 120 V Inside Frost, Philips Electronics, Scarborough, ON, Canada) housed in a light box constructed from white Coroplast® (Boyds, Kelowna, BC, Canada) positioned 13 cm above the tunnel. The mean light intensity at the point of moth release and odor plume height was 1.8 lx (Minolta Auto Meter IVF). Room lights remained off for 22 h at which time the assay was concluded, trapped moths were counted, and nonresponders were vacuumed from the tunnel in time to prepare for the next assay protocol at 1000 hours. Air moved through each tunnel at 30–40 cm s^{-1} and was exhausted directly to the roof of the building. All experiments were conducted at 22–24°C.

Assay protocol On each of the five assay days (replicates), 34–75 moths, depending upon moth availability, were placed in a flight tunnel. The numbers of moths caught in each of two traps during the next 22-h assay period were recorded, and the percentages of moths in each trap and the number recaptured out of the total released during each replicate were calculated.

Lures Acetic acid was dispensed from 15 ml polypropylene bottles (Nalg-Nunc International, Rochester, NY, USA). Each bottle contained 10 ml of glacial acetic acid (Aldrich Chemical, Milwaukee, WI, USA) applied to cotton balls in the bottom of the bottle. Volatilized acetic acid was emitted through a 1-mm diameter hole in the bottle lid (Landolt and Alfaro 2001). Pear ester (Aldrich Chemical, Milwaukee, WI, USA) was dispensed from gray halobutyl rubber septa (West, Lyonville, PA, USA) with a lure load of 100 μg pear ester and 10 mg butylated hydroxytoluene (BHT; Aldrich Chemical, Milwaukee, WI, USA; as an antioxidant) per septum. Septa were preextracted with methylene chloride and were then aired for 24 h at 24°C in a fume hood before use. Loaded septa were stored in a freezer at 0°C between experiments. Upright bottles and septa were attached to either end of a 10-cm long wire bent to the shape of a horseshoe, such that they were separated by 1 cm in the same horizontal plane. Lures were hung by this wire in the geometric center of each trap with the septum always located directly downwind of the bottle's opening such that volatilized acetic acid could drift over the septum.

Experimental design Five dual-choice laboratory experiments (experiments 1–5) were conducted and repeated separately with virgin males, virgin females, and mated females. All insects used in these experiments had no prior exposure to test chemicals. In experiment 1, we quantified the catches of moths in two paired, blank traps placed upwind, thus revealing any upwind flight tendencies of the different moths and potential positional trap biases. Experiments 2–4 quantified the attraction of moths to acetic acid and pear ester alone and in combination, respectively, relative to a blank trap in paired comparison tests. In these experiments, blank traps were provided with empty bottles, clean septa, or both, respectively. In experiment 5, we compared the relative attractiveness of the combination of acetic acid plus pear ester vs. pear ester alone.

Moth catches were expressed as proportions (p) and transformed by using an arcsine-$\sqrt{p}$. Mean proportions caught (N=5) were compared by using a paired t test. The mean total proportions of virgin male, virgin female, and mated female moths caught during each dual-choice experiment were compared by using an analysis of variance (ANOVA) followed by Tukey's multiple comparisons test with the experiment-wise error rate set at α=0.05 (Zar 1984). All statistical analyses were performed with SigmaStat® version 3.0.1 (SYSTAT Software, San Jose, CA, USA).

Field Experiments

Three trapping experiments evaluated CM responses to four treatments: no chemical lure as a control, acetic acid, pear ester, and the combination of acetic acid with pear ester. Acetic acid was dispensed from a 15-ml polypropylene bottle with a 3-mm diameter hole in the lid. Each bottle was loaded with 10 ml of glacial acetic acid on cotton balls. Pear ester was dispensed from gray rubber septa, preextracted, and handled as described above. Each septum was loaded with 200 μl of a solution of 1 mg of pear ester and 10 mg of BHT in methylene chloride.

The first experiment, conducted near Zillah, Yakima County, Washington, USA, used Universal Moth Traps (AgriSense, Norwich, UK). A 6-cm^2 piece of Vaportape (Hercon Environmental, Emigsville, PA, USA) was stapled to the inside of the trap bucket to kill captured moths. Acetic acid lures were suspended upright within the buckets of the traps, and pear ester lures were placed within the lure baskets of the traps at the center of the underside of the trap lid. A randomized complete block design was used with 10 block replicates set up in a commercial apple orchard on August 24, 2006. Traps were checked weekly for 2 wk. The orchard had been treated with codling moth pheromone for mating disruption.

The second experiment was conducted also near Zillah and used the Pherocon 1C trap (Trece, Adair, OK, USA). These traps have a white plastic roof and floor separated by an open space for entry of moths with a sticky liner as the trap floor. Acetic acid dispensers were placed upright in the adhesive at the center of the traps' sticky liners. Each pear ester septum was pinned to a 6-cm^2 piece of cardboard placed in the trap liner adhesive. A randomized complete block design was used with 10 replicates. This was set up in a commercial apple orchard that had been treated with CM pheromone on September 12, 2006. Traps were checked weekly for 2 wk.

The third experiment was conducted near Lincoln, the South Island of New Zealand. Red Delta traps (HortResearch, Aukland, NZ) were baited with the same lures used in the previous two field experiments. The Delta trap is a tent-shaped trap open at two ends allowing entry of moths with a sticky liner in the bottom of the trap (Clare et al. 2000). Lures were placed upright at the center of the trap liner. Ten blocks were set up in an experimental apple orchard on November 28, 2006, and traps were maintained for 4 wk.

Mean numbers of moths captured were compared between treatments by using ANOVA, and following a significant F value, means were separated using Tukey's test at $\alpha<0.05$.

Results and Discussion

Laboratory Experiments

In experiment 1, blank (unbaited) traps surprisingly caught large and similar numbers of virgin males and mated females. However, fewer virgin females than virgin males or mated females were in blank traps (Table 1). Most virgin females appeared to be stationary and engaged in "calling" behavior immediately after the lights went out, whereas many mated females and virgin males repeatedly made upwind excursions and eventually landed in the traps.

In experiment 2, acetic acid was not attractive (Table 1), although the response by mated females was nearly significant ($P=0.051$). Pear ester lures in experiment 3 were attractive to both males and mated females (Table 1), consistent with field reports (Light et al. 2001), but not to virgin females. The combination of acetic acid and pear ester in experiment 4 was attractive to all three categories of moths with the greatest percentage response by mated females (Table 1).

The percentages of males caught per test were similar across laboratory tests, irrespective of the treatment comparison. However, captures of mated and virgin females were greater in experiments where acetic acid plus pear ester was evaluated. This suggests that more of the females engaged in upwind flight when this lure was in the flight tunnel. We speculate that virgin males may engage in anemotactic flights even in the absence of relevant odor cues, but will then likely follow the plume of a relevant attractant to its source, depending on their "motivational" state. Collectively, these results support the hypothesis that this lure elicits a food-finding response by males and mated females. Virgin females are perhaps "motivated" to find a mate before feeding.

In experiment 5, males, mated females, and virgin females were all more attracted to the combination lure than to pear ester alone. A positive interaction, possibly synergy, of acetic acid and pear ester was most clearly demonstrated in the responses of virgin females to this lure, because neither lure was more attractive than a blank when tested alone, but a significant response was shown to the combination lure (Table 1).

Field Experiments

In all three field experiments, both female and male CM were more strongly attracted to (trapped with) the combined lure than to either individual component (Table 2), consistent with the results of laboratory tests. This enhanced response to the combined lure was more than 200% higher compared with either compound alone. Acetic acid alone attracted no more CM than unbaited traps, and only in the New Zealand test were numbers of CM trapped with pear ester higher than the controls (Table 2). There are no prior reports of codling moth attraction to acetic acid with pear ester, combinations of other nonpheromonal chemicals with pear ester, or other chemicals with acetic acid.

Codling moth has been trapped with acetic acid (Yothers 1927; Eyer 1931; Dethier 1947) and with pear ester (Light et al. 2001). However, in this study, there was no attraction to acetic acid. Perhaps, a significant response would have been observed with a different acetic acid release rate, higher moth densities, or with more replications. Knight and Light (2004) in Washington captured both sexes with pear ester; but in other studies, mostly

Table 1 Flight-tunnel choice tests comparing attractiveness of different lures to naive 2-d-old adult codling moths from the Okanagan–Kootenay, mass-rearing colony, Osoyoos, BC, Canada, February–April, 2007

Exp. No.	*N*	Paired Lure Choices[a]	Mean±SE % Moths Caught/Trap/Lure Type[b]			Mean±SE total % of moths caught/experiment[c]		
			Virgin males	Virgin females	Mated females	Virgin males	Virgin females	Mated females
1	5	Blank	44.1±5.8 ns	54.5±8.5 ns	51.0±3.3 ns	36.6±2.4a	16.5±2.3b	42.4±3.5a
		Blank	55.9±5.8	45.5±8.5	49.0±3.3			
2	5	AA	50.7±5.9 ns	54.1±3.3 ns	61.4±4.2 ns	40.7±2.6b	21.9±3.1c	51.7±2.4a
		Blank	49.3±5.9	45.9±3.3	38.6±4.2			
3	5	PE	63.9±5.6 *	49.6±4.3 ns	69.5±3.4**	42.4±2.1a	22.7±5.4b	48.0±0.9a
		Blank	36.1±5.6	50.4±4.3	30.5±3.4			
4	5	AA+PE	76.2±4.7**	72.9±1.8***	80.3±1.7***	43.1±0.8b	31.1±4.3c	62.2±2.2a
		Blank	23.8±4.7	27.1±1.8	19.7±1.7			
5	5	AA+PE	67.5±4.0 *	62.7±3.4*	71.9±2.6***	49.9±4.6b	20.3±2.3c	60.1±6.3a
		PE	32.5±4.0	37.3±3.4	28.1±2.6			

Males were tested in the push flight tunnel and females in the pull flight tunnel.

[a] AA=15 ml of glacial acetic acid in propylene bottles with single 1 mm hole in lid; PE=100 μg load of ethyl-(*E*,*Z*)-2,4-decadienoate, pear ester, on gray halobutyl rubber septa lures

[b] Significance of paired *t* tests comparing paired mean percentages of catches with different lures and within an experiment are indicated by: ***=$P<0.001$; **=$P<0.005$; *=$P<0.05$; ns=$P>0.05$; $N=5$.

[c] Mean total percentages of moths caught in treatment and control traps combined for each experiment. Total number of moths released in each flight tunnel for each replicate of each experiment (1–5) ranged from 34 to 75 moths. Mean total percentages within a row (experiment) followed by different letters are significantly different (Tukey's test $\alpha<0.05$) following significant ANOVA ($P<0.05$).

Table 2 Mean numbers of male and female codling moths captured in traps baited with acetic acid (AA), ethyl-(*E*,*Z*)-2,4-decadienoate (PE), or the combination of acetic acid and ethyl-(*E*,*Z*)-2,4-decadienoate (AA+PE)

	UniTrap, WA	Wing Trap, WA	Delta Trap, NZ
Female codling moths			
Blank	0.0+0.0a	0.0+0.0a	0.0+0.0a
AA	0.4+0.2a	0.3+0.2a	0.2+0.1a
PE	0.2+0.1a	0.2+0.1a	6.0+1.2b
AA+PE	2.2+0.7b	0.8+0.4b	16.9+2.2c
Male codling moths			
Blank	0.0+0.0a	0.0+0.0a	0.0+0.0a
AA	0.5+0.3a	0.2+0.1a	0.9+0.3a
PE	0.3+0.2a	0.2+0.1a	15.5+3.2b
AA+PE	2.3+0.7b	0.7+0.3b	32.6+3.3c

Tests were conducted in Washington (WA) and in New Zealand (NZ). Means within a column followed by the same letter are not significantly different by Tukey's test at $\alpha<0.05$.

males (Il'ichev 2004; Thwaite et al. 2004) or only males were trapped (Kutinkova et al. 2005). Our results from Washington did not find a response by either sex to pear ester, but those from New Zealand showed responses by both sexes, as did our laboratory tests with virgin males and mated females. Codling moth attraction to pear ester varies with season, pheromone treatments for mating disruption, orchard type, and other parameters (Knight et al. 2002; Il'ichev 2004; Knight and Light 2005c; Kutinkova et al. 2005; Trimble and El-Sayed 2005). A significant response to pear ester may have been observed in our field tests in Washington under different conditions, such as higher moth populations.

Codling moths may respond to pear ester with acetic acid to locate adult food. The possibility that CM orient to ripe fruit and to acetic acid plus pear ester as food finding behavior does not exclude the additional possibility that their attraction to fruit odorants, such as pear ester, is also a means to locate oviposition sites or potential mates. Indeed, females are stimulated to lay eggs in the presence of the ripe fruit odorants *E*,*E*-α farnesene (Sutherland et al. 1974) and pear ester (Knight and Light 2004).

It remains to be determined if acetic acid enhances attraction of CM to other fruit odorants, such as those emitted from ripe apples. Several compounds emitted by mature or ripe apple fruit have been identified, are antennally and behaviorally active, but have not been demonstrated to be attractive in the field (Hern and Dorn 1999, 2001, 2004; Yan et al. 1999; Backmann et al. 2001; Bengtsson et al. 2001; Vallat and Dorn 2005). Tests should be conducted to determine if any of these or a blend of apple odorants are coattractive with acetic acid. Codling moths are attracted to ripe 'Braeburn' apples, although less so than to ripe 'Bartlett' pears (Landolt and Guédot 2008), and the attractive chemicals from those apples have not yet been isolated.

Our results hold promise for improving the power of attraction of nonpheromonal lures for CM with a greater than twofold increase in both sexes trapped with acetic acid with pear ester. Where pear ester has shown promise as a management tool for CM, on apple, walnut, and pear crops (Knight and Light 2004, 2005a, b), this lure might improve the efficacy of monitoring, attract-and-kill, or mass trapping. Where the response of females to pear ester has been weak or nonexistent (Kutinkova et al. 2005), perhaps an improved response would be obtained with acetic acid with pear ester, as was shown for both Washington and New Zealand.

Acknowledgments Technical assistance was provided by Daryl Green in Washington, USA; Vanessa Mitchell in New Zealand; and Mark Gardiner and Sarah Cockburn in British Columbia, Canada. This work was supported by grants from the Washington State Tree Fruit Research Commission and the New Zealand Foundation for Research Science and Technology, CO6X0301, Insecticide Risk Reduction.

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valuable levels of resistance. A screening of seven sunflower genotypes in a field experiment showed a correlation between these compounds and resistance to *Sclerotinia*.

Keywords Acetophenones · Ayapin · Demethoxyencecalin · Scopoletin · Phenolics · Coumarins · Sunflower · Bracts · *Sclerotinia sclerotiorum* · Allelochemicals

Introduction

Sunflower (*Helianthus annuus* L.) represents an important crop that is a source of oils, high-protein meal, birdseed, and ornamental flowers. *Sclerotinia sclerotiorum* (Lib.) de Bary is a worldwide distributed sunflower pathogen that attacks most parts of the plant, including the root, stem, capitulum, leaf, and terminal bud, at any developmental stage. This disease is of special virulence in Argentina, France, and Japan (Acimovic 1985). Under favorable climatic conditions, high humidity and mild temperature, the fungus causes yield losses, especially in the case of capitulum infection (head rot disease) (Purdy 1979). Losses caused by *Sclerotinia* vary according to the maturation stage of the capitula at the onset of infection. The period of maximum susceptibility is at the onset of flowering (when the outermost and next inner radii of florets reach anthesis), causing losses of up to 100% at this stage (Purdy 1979).

Head rot starts when spores, transported by the wind, reach a healthy sunflower capitulum. Under high humidity, spores germinate and penetrate into the flowers, thus spreading to the bracts and receptaculum (Pereyra et al. 1991), and degrading the capitulum. As the fungus degrades tissues, mycelia accumulate and form sclerotia that fall into the soil where they can survive up to the following crop season when sclerotia germinate and the apotecia liberate the new spores, thus starting a new disease cycle. The isolation and structural characterization of defense compounds in resistant varieties is an important goal of plant breeders who are pursuing crop improvement. Moreover, the development of easy analytical methods for chemical detection would provide a powerful tool for observing the transfer of resistance traits during the breeding process. As chemical control is not practical, genetic control through the development of resistant lines appears to be the best crop protection strategy. Accordingly, a major goal of research is to identify compounds that are involved in resistance, to use them as biochemical markers in plant breeding programs, or to design appropriate control strategies.

Sunflower constitutes a rich source of biologically active secondary metabolites with a wide spectrum of activities (Macías et al. 1999). Many are specifically located in the trichome glands (Spring et al. 1992, 2003), and it has been hypothesized that many play a role in chemical defense. Some have proved their effectiveness as insecticidal, e.g., against the sunflower fly *Homoesoma ellectellu*m (Waiss et al. 1981) or *Diabotrica virgifera* (Mullin et al. 1991), fungicidal (Picman and Schneider 1993), or allelopathic (Macías et al. 1998) agents.

In a previous study, we assessed the resistance of a large number of sunflower genotypes to *Sclerotinina* head rot (Prats et al. 2003). Based on differences in susceptibility, four of these were selected for detailed analysis. The resistant genotype not only contained the highest constitutive phenylalanine ammonia-lyase (PAL) activity and total soluble phenolic content, but *S. sclerotiorum* infection also induced PAL activity, and consequently, the synthesis of phenolics (Prats et al. 2003). By contrast, the most susceptible genotype showed a relatively low constitutive PAL activity level and phenolic content, and there was no disease-induced response. We further observed high correlations between coumarins (especially scopolin and ayapin) and *Sclerotinia* resistance (Prats et al. 2006). From thin

layer chromatography (TLC) analyses, it appeared that other phenolics were also more abundant in the resistant genotype. The present study extended the previous work by isolation of the compounds that may be involved in *Sclerotinia* head rot resistance.

Methods and Materials

General methods Solvents were glass-distilled or commercial HPLC grade. Column chromatography was performed on silica gel (35–75 mesh), and TLC analysis was carried out by using aluminum-packed precoated silica gel and Silicagel 60 F_{254} plates (Merck). For HPLC, LiChrosorb silica 60 was used in the normal-phase mode with a differential refractometer (RI) on a Hitachi L-6020 HPLC instrument. ^{1}H NMR and ^{13}C NMR were recorded on a Varian Unity 400 spectrometer with $CDCl_3$ as solvent. The resonances of residual chloroform at δ_H 7.25 and δ_c 77.0 were used as internal reference for ^{1}H NMR and ^{13}C NMR spectra. Mass spectra were obtained with a VG 1250 AUTOSPEC instrument at 70 eV. The IR spectra were recorded on a BioRad FTS-7. UV spectra were recorded on an HP 8453 UV/Vis spectrophotometer.

Plant material Sunflower genotypes were provided by Advanta Seeds S.A.I.C. (Balcarce, Argentina). For the isolation and characterization of new compounds, a 2,500-m^2 experimental field in Balcarce, Argentina was sowed with genotype 1V8410. Sunflower inner bracts from each plant were collected 14 d after flowering when the outermost and next inner radii of florets reached anthesis (maximum susceptibility stage).

To test the relation between the new compounds and resistance to *S. sclerotiorum in vivo*, a set of seven sunflower experimental genotypes, different from the genotype used for isolation and purification of phenolics, was sowed in an experimental field. Each plot consisted of two rows, which are 6 m long and 0.7 m apart (20 plants per row). For each genotype, five plants were randomly chosen to assess constitutive phenolic content. Bracts were harvested 14 d after the beginning of flowering, frozen in liquid nitrogen, and maintained at −80°C before analysis. At beginning of flowering, between 7 and 16 plants of similar flowering date were inoculated artificially by spraying with 1.5 ml of a water suspension of ascospores (20,000 ascospores ml^{-1}). To ensure high humidity conditions with no thermal stress, plants were covered with a paper bag (Prats et al. 2003). Thirty days after inoculation, the percentage of necrosed capitulum was determined. Necrotic lesions in the receptacle were quantified using a discontinuous scale from 0% (no necrotic lesions observed) to 100% (tissue totally necrosed) with intermediate values of 10, 25, 50, and 75, indicating the percentage of the total receptacle affected. For each genotype, bracts of five randomly chosen plants were harvested 14 d after inoculation to assess phenolic content.

Isolation of compounds Fresh plant material was lyophilized (438 g) and ground in a mortar with a mixture of acetone/methanol 5:1 (*v*/*v*) at room temperature. After filtration, the extract was evaporated under vacuum (40°C) to yield 24 g of crude extract. The extract was fractionated by column chromatography on silica gel by using mixtures of hexane/diethyl ether of increasing polarity, yielding 164×50 ml fractions. TLC analysis of the crude extract with different hexane/diethyl ether mixtures as mobile phases allowed us to group the crude extract into six fractions (I–VI). The coumarins ayapin (**1**), scopoletin (**2**), and scopolin (**3**) were detected as fluorescent spots under the 254-nm wavelength by comparison of their Rf values with pure samples. Further HPLC purification of fractions I,

IV, and VI with a UV detector monitored at 254 nm wavelength afforded compounds **4** (19 mg), **5** (12.2 mg), and **6** (1.7 mg), respectively.

In vitro bioassay To check the inhibitory effect of the different isolated compounds, a quantitative test for *Sclerotinia* growth inhibition was performed in flat-bottomed microtiter plates. Different concentrations (0.025, 0.05, 0.075, and 0.1 μg equivalent of chlorogenic acid) of 3-acetyl-4-hydroxyacetophenone (**6**), demethoxyencecalin (**4**), 3-acetyl-4-acetoxyacetophenone (**5**), and ayapin (**1**) in methanol were vacuum-dried. The residue was redissolved by repeated aspiration with a pipette in 100 μl of Czapek–Dox broth (Sigma) containing *Sclerotinia* ascospores (2×10^4 ml^{-1}). Plates were incubated for 18 h at 21°C with continuous illumination (4 μEm^2 s^{-1}). The lengths of 15 ascospore germ tubes were measured in each of the 3 replicates of each treatment using an inverted white light microscope (CK2 Olympus, ×100).

TLC assay TLC plates were used to visualize and semiquantify the phenolic content of the seven experimental genotypes. Bracts were extracted as reported in Prats et al. (2003). Frozen tissue was homogenized with prechilled (−20°C) 1:10 (*w*/*v*) acetone in a mortar. After filtering the solvent, the residue was further sequentially extracted with a similar volume of acetone and acetone/methanol (1:1 *v*/*v*). The combined organic extracts were concentrated under vacuum, redissolved in a minimum volume of methanol, and applied to a TLC plate. Ethylacetate was used as the mobile phase. Plates were visualized under UV light (254 nm), and compounds were identified according to Rf values. The content of each compound was estimated visually according to a 0–5 scale from the lowest to the highest intensity and transformed to a 0–100 scale for easy comparison with disease levels.

Results

Previous analysis from *Sclerotinia* resistant sunflower genotype (1V8410) demonstrated that bract and corolla extracts contained high amounts of the known coumarins ayapin (**1**), scopoletin (**2**) and its glucoside derivate, scopolin (**3**) (Prats et al. 2006), and three other unknown compounds **4**, **5**, and **6** with Rf values of 0.7, 0.6, and 0.4, respectively. These showed violet fluorescence under the 254-nm UV light in the TLC analysis (Prats et al. 2003). TLC showed low concentrations of these compounds in the susceptible genotype (cv.30302) and moderate concentrations in the moderately resistant genotypes (FRTS and 30145) (Prats et al. 2003). Two were identified by means of their spectroscopical data (^{1}H NMR and ^{13}C NMR, UV, IR, and MS) as the already known compounds demethoxyencecalin (**4**) and 3-acetyl-4-hydroxyacetophenone (**6**). Compound **5** was identified as 3-acetyl-4-acetoxyacetophenone (Fig. 1). This compound presented a molecular ion in the HRMS at *m*/*z* 220.22213 corresponding to a molecular formula $C_{12}O_4H_{12}$. The ^{1}H NMR spectra was similar to that of compound (**6**), being characterized with the presence of an ABX system in the aromatic zone (δ 8.31, δ 8.27, and δ 7.25); two aromatic methyl groups at δ 2.61 and δ 2.39 (3H, s, H-10), and one acetoxyl group at δ 2.14.

*3-Acetyl-4-acetoxyacetophenone (***5***)* IR: ν_{max} (KBr) cm^{-1}: 1740 (ester group), 1650 (carbonyl group), 1614 (carbonyl group); HRMS $[M]^+$ 220.22213, $C_{12}O_4H_{12}$ requires

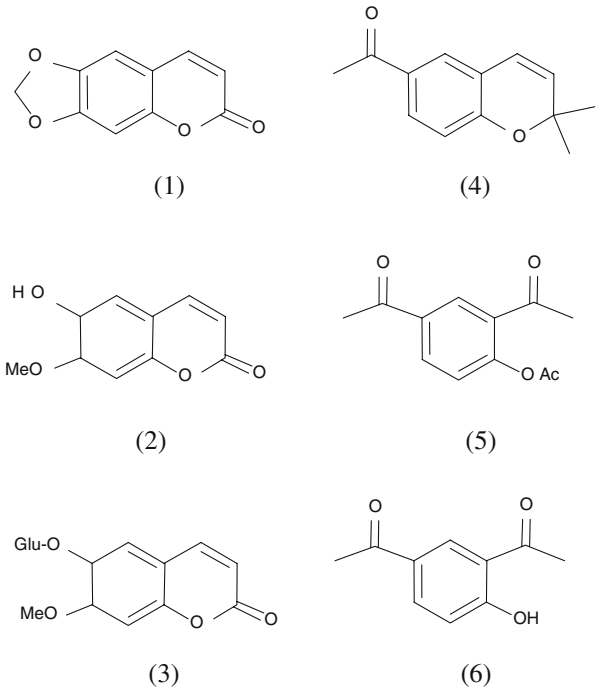


Fig. 1 Fungicidal compounds isolated from *Sclerotinia* resistant sunflower bracts. Ayapin (**1**), scopoletin (**2**), scopolin (**3**), demethoxyencecalin (**4**), 3-acetyl-4-acetoxyacetophenone (**5**), 3-acetyl-4-hydroxyacetophenone (**6**)

220.07356; UV $\lambda_{max}^{CHCl_3}$nm: 235.3, 290.0; ^{1}H NMR (400 Mz, $CDCl_3$): δ 2.14 (3H, s, –OCO–*CH*$_3$); δ 2.39 (3H, s, H-10); δ 2.61 (3H, s, H-8); δ 7.25 (1H, *d*, *J*=8.7 Hz, H-5); δ 8.27 (1H, *dd*, *J*=8.7 Hz, *J*=2.0 Hz, H-6); δ 8.31 (1H, *d*, *J*=1.9 Hz, H-2). Fluorescence: 290 (λEx); 412 (λEm).

An *in vitro* bioassay carried out with **4**, **5**, **6**, and ayapin as a known potent *Sclerotinia* inhibitor showed that 3-acetyl-4-hydroxyacetophenone (**6**) had the highest inhibitory effect against *Sclerotinia*, followed by demethoxyencecalin (**4**). The new compound, 3-acetyl-4-acetoxyacetophenone (**5**), was also a potent *Sclerotinia* inhibitor with its activity being in the same range as that observed for ayapin (**1**) (Fig. 2).

Data from the field experiment showed a significant (P=0.022) correlation between the total amount of these three compounds and disease resistance, thus supporting the hypothesis of an *in vivo* inhibitory effect. Genotype CP03-1790, which had the lower percentage of necrosed capitulum, showed the highest amounts of **4**, **5**, and scopoletin, and was the only genotype with **6** (Table 1). This was true for both noninoculated and inoculated plants. An overall analysis of all genotypes showed that these compounds did not significantly increase after inoculation. Ayapin tended to increase after inoculation in most genotypes, however, the increase was not significant. Genotypes CP03-1791, CP03-1792, and CP03-1793 showed intermediate resistance with necrosed values ranging from 4.2% to 43%, and also had intermediate values of the compounds. Genotypes CP03-1794, CP03-1795, and CP03-1796 showed the highest disease values with more than 60% necrosed capitulum. This was associated with the lowest amount of phenolic compounds (Table 1). When analyzed individually, only **4** and **5** showed significant correlations with disease resistance (P=0.03).

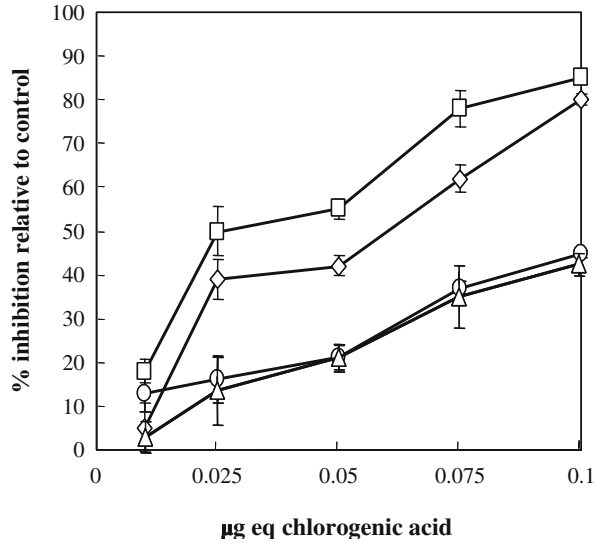


Fig. 2 Effect of 3-acetyl-4-acetoxyacetophenone (**5**), 3-acetyl-4-hydroxyacetophenone (**4**), demethoxyencecalin (**3**), and ayapin (**2**) on *Sclerotinia* development. *Lines* represent the percentage of inhibition of different compounds respect control (spores incubated only in Czapek–Dox broth medium). *Bars* indicate 95% confidence intervals. *Open squares* 3-acetyl-4-hydroxyacetophenone, *open diamonds* demethoxyencecalin, *open triangles* 3-acetyl-4-acetoxyacetophenone, *open circles* ayapin

Discussion

Sunflower produces phenolic compounds from the phenylpropanoid biosynthetic pathway (reviewed by Dixon et al. 2002), which is regulated by the conversion of phenylalanine to *trans*-cinnamic acid by the enzyme PAL (Bate et al. 1994). Among phenolics, sunflower coumarins have attracted attention because of evidence that their biosynthesis is induced by abiotic stress (Cabello-Hurtado et al. 1998) and by the attack of the parasitic plant *Orobanche* (Serghini 2000) and pathogenic fungi (Ding et al. 2000; Prats et al. 2002). In particular, ayapin and scopoletin inhibit *S. sclerotiorum* growth (Urdangarin et al. 1999), and scopolin has antifungal activity similar to scopoletin (Prats et al. 2006).

To our knowledge, this is the first report of phenolic compounds, other than the known coumarins, with high inhibitory effects against *S. sclerotiorum*. Demethoxyencecalin (**4**) is a chromene that was previously isolated from other species (Becerra et al. 1983; Kulkarni et al. 1987) and *Helianthus annuus* (Varga et al. 1984; Satoh et al. 1996). It was isolated from the stem and receptaculum. Demethoxyencecalin, along with other chromenes, has been described as an insect feeding deterrent against *Phythorimaea operculella* and *Spodoptera litoralis* (Kulkarni et al. 1987; Srivastava and Proksch 1993). This compound is also fungicidal, bactericidal, and possesses antioxidant activity (Okunade et al. 1997; Tsuda et al. 1997). Isolation from resistant sunflower varieties, thus, is in agreement with a proposed protective role. 3-Acetyl-4-hydroxyacetophenone (**6**) has been isolated previously from *Artemisa campestris* subsp. *glutinosa* (De Pascual et al. 1981), but there are no previous references to biological activity. In this study, we showed that this compound has fungicidal activity. Indeed, the *in vitro* assay showed that **6** had the highest inhibitory effect on *Sclerotinia* growth. Furthermore, *in vivo* experiments showed that the only genotype containing this compound, CP03-1790, had the lowest disease level. It is interesting to note that the genotype 1V8410, which was used for the isolations and characterizations, showed high levels of 3-acetyl-4-hydroxyacetophenone (**6**) and less than 1% of necrosed capitulum. The new compound 3-acetyl-4-acetoxyacetophenone (**5**) also showed a high *in vitro*

Table 1 Relative content of phenolic compounds extracted from bracts of noninoculated and inoculated sunflower genotypes and percentage of necrosed capitulum assessed 30 d after inoculation

Genotype	Non-inoculated					Inoculated					
	Dem[a]/Ac[b]	Ayapin	Hydr[c]	Scopoletin	Total	Dem/Ac	Ayapin	Hydr	Scopoletin	Total	% Necrosis
CP03-1790	68±13.5	nd	16±4.0	42±11.1	126±26.0	40.0±13.9	4.0±2.6	4.0±1.6	14.0±7.8	62.0±22.4	1.1±1.0
CP03-1791	12±4.8	nd	nd	10±0.0	22±4.8	16.0±2.4	nd	nd	nd	26.0±2.4	18.0±8.5
CP03-1792	10±0.0	0.5±0.0	nd	nd	10.5±0.0	10.0±4.4	10±0.0	nd	nd	20.0±4.4	4.2±2.9
CP03-1793	16±6.0	0.3±0.1	nd	nd	16.3±5.9	28.0±18.5	4.0±2.4	nd	nd	32.0±19.8	43.3±10.4
CP03-1794	6±4.0	0.7±0.5	nd	nd	6.7±4.5	6.2±2.6	3.8±1.8	nd	nd	10.0±3.7	100±0.0
CP03-1795	nd	0.1±0.1	nd	nd	0.1±0.1	nd	nd	nd	nd	nd	67.3±6.5
CP03-1796	2±1.8	0.8±0.5	nd	nd	2.8±2.5	4.3±2.9	4.3±2.0	nd	nd	8.6±4.0	60.7±12.6

Content of each compound was estimated visually from a TLC plate according to a 0–5 scale from the lowest to the highest intensity and transformed to a 0–100 scale for easy comparison with disease level. Values of demethoxyencecalin and 3-acetyl-4-acetoxyacetophenone with close Rf values were identical in all cases and are expressed as Dem/Ac. Data are the mean of at least five plants and are expressed as mean±standard error.

nd: not detected

[a] Demethoxyencecalin (**4**)

[b] 3-acetyl-4-acetoxyacetophenone (**5**)

[c] 3-acetyl-4-hydroxyacetophenone (**6**)

inhibitory effect on *Sclerotinia*, similar to that of ayapin, and was also significantly correlated with reduced disease levels *in vivo*.

It is important to point out that the compounds are localized on the bracts and corollas where the most virulent attack of the pathogen is confined, and also their constitutive nature. Previous results have shown low coumarins and total phenolic accumulation in vegetative organs compared to those observed in bracts and corollas (Prats et al. 2003, 2006). Head rot infection usually starts in bracts and corollas; therefore, these compounds there represent a first defense barrier against infection. We did not observe an increase after inoculation. However, in previous work, we observed a slight significant increase of coumarins after inoculation in resistant genotypes, which is supported by the increase in PAL activity (Prats et al. 2003, 2006). Although visual evaluation of TLC plates allowed us to discriminate susceptible and resistant genotypes, this method may not be accurate enough to detect slight changes after inoculation. In general, *Sclerotinia* infection proceeds rapidly, so that in susceptible genotypes, the capitula are highly colonized within 14 d. In such cases, high constitutive coumarin/phenolic concentrations coupled with *de novo* synthesis in response to attack might lead to a sufficient accumulation to inhibit the fungus. This might not be possible only with *de novo* production. Previous work also has demonstrated that sunflower coumarins, produced constitutively or after treatment with a systemic inducer of resistance, inhibit rust appressorium formation (Prats et al. 2002, 2007).

Coumarins and other phenolic compounds can be detected and semiquantified rapidly with TLC preparations under UV light (254 nm). HPLC/diode array detection and quantification methods are also easy to develop. Thus, the compounds may be useful markers for head rot resistance in operational sunflower plant breeding programs. The speed and simplicity of the detection approaches make them suitable as screening procedures for identifying resistant genotypes.

Acknowledgement This work was supported by the Spanish Ministry of Science and Technology, Project AGL 2001-2420.

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frequency on root surfaces and fungal sensitivity to totarol. We conclude that totarol is one factor that selects for fungi on root surfaces of *T. dolabrata* var. *hondai* in the early growth stage.

Keywords *Thujopsis dolabrata* var. *hondai* · Seed fungi · Soil fungi · Rhizosphere · Antifungal compound · Totarol

Introduction

Hinoki-asunaro (*Thujopsis dolabrata* Sieb. et Zucc. var. *hondai* Makino; also called hiba or ate) is an endemic tree in Japan. It is distributed from the Oshima Peninsula (42°10′N) on Hokkaido to Tochigi Prefecture (36°47′N) on Honshu (Kobayashi and Asakawa 1981). The seeds (Hasegawa and Hirose 1980), leaves (Takahashi et al. 1981; Nagahama et al. 1996), trunk, and branches contain characteristic secondary metabolites. In particular, essential oil extracted from *T. dolabrata* var. *hondai* wood, which contains sesquiterpenes and hinokitiol-related compounds, has antimicrobial activity against bacteria (Okabe et al. 1994; Saito et al. 1997; Morita et al. 2001), wood-rotting fungi (Inamori et al. 2000), and plant pathogens (Morita et al. 2004), and is insecticidal against termites (Miyazaki 1996; Lee et al. 1997; Ahn et al. 1998). Houses made of the timber are free of decay and infestation for a long period, thus making *T. dolabrata* var. *hondai* a commercially important tree in Japan.

Seedlings show physiologically and ecologically characteristic germination. Because of slow growth, several nursing systems for *T. dolabrata* var. *hondai* have been developed (Aoki 1936; Fujimori 1958; Sakurai and Mori 1985a, b; Itoya and Shimoda 1995, 1999; Nakano 2000; Mori et al. 2003; Aomori Forestry Research Institute 2004). In naturally regenerated forests, seedlings grow in mineral soil without humus (Mori et al. 2003). Tohoku Research Center of the Forestry and Forest Products Research Institute (FFPRI) in Japan has developed a nursing system that imitates conditions in natural forests. The seedlings require 6–30% relative light intensity to germinate and grow, although *T. dolabrata* var. *hondai* is a shade-tolerant tree (Sakurai and Mori 1985a, b; Itoya and Shimoda 1995). Therefore, the nursery bed is set 20 cm lower than the surrounding ground and is covered with plastic shading nets (Itoya and Shimoda 1999). Instead of the humic nursery soil generally used for the seedbed (Aomori Forestry Research Institute 2004), Kanuma pumice is used as a model mineral soil of the naturally regenerated forest (Mori et al. 2003).

Plant roots interact with symbiotic, pathogenic, or epiphytic microorganisms in the rhizosphere, and root exudates (amino acids, nucleotides, sugars, and secondary metabolites) influence microbial populations in the rhizosphere (Brimecombe et al. 2001). Flavonoids and isoflavonoids exuded from roots act as organic signals in legume–rhizobia symbiosis (Werner 2001). For instance, species of *Rhizopus* and *Fusarium* in the rhizosphere of Brassicaceae plants are selected by secondary metabolites (isothiocyanates) exuded from the roots (Ishimoto et al. 2000). Seeds of *T. dolabrata* var. *hondai* have resin cavities on the seed surface and contain several diterpenes and lignans (C_6C_3 dimers; Hasegawa and Hirose 1980), and terpenoids and phenolics that are generally antifungal (Waterman and Mole 1994). Nursery beds of *T. dolabrata* var. *hondai* smell like terpenoids after germination, and germination gradually continues for 5 mo after sowing (Sakurai and Mori 1985a; Itoya and Shimoda 1995; K. Yamaji, unpublished data).

Based on these previous findings, we hypothesized that antifungal compounds of seeds might select for fungi on root surfaces of *T. dolabrata* var. *hondai* seedlings in the early growth stage, i.e., antifungal compounds of seeds might influence seed and soil fungi, and

those seed and soil fungi tolerant of antifungal compounds might grow on seedling root surfaces. In this study, we examined seedlings growing in Kanuma pumice and nursery soil because different soil fungi and nutrient conditions may influence which fungi will grow on the root surfaces. We identified seed and soil fungi and examined the sensitivity of these to methanol extracts of the seeds. In addition, we identified fungi on the seedling root surfaces and calculated the fungal detection frequency (FDF, %). We then examined the relationship between FDF on root surfaces and the fungal sensitivity to the seed extract. Finally, we isolated from the seeds the antifungal compound totarol, and tested it for antifungal sensitivity.

Methods and Materials

Seeds, Soil, and Seedlings *Thujopsis dolabrata* var. *hondai* seeds were randomly collected from approximately 20 felled trees in natural forests in Nakasato, Aomori Prefecture, northern Honshu, Japan (40°58′N, 140°28′E) in October 2002. *T. dolabrata* var. *hondai* trees have good, normal, and poor harvest years, and the harvest (3.23 g fresh weight [FW], 1000 seeds) was poor in 2002. Seeds were kept at 4°C in a paper bag until use.

Kanuma pumice, a volcanic andesitic rock and highly weathered, was purchased (Kyodo-Jirushi, Kanuma, Japan). Fifteen 18-l bags of Kanuma pumice were mixed uniformly and used for the experiments. Humic nursery soil that has andic and allophanic properties was collected in a *T. dolabrata* var. *hondai* nursery in Hiranai (50×100 m), Aomori Prefecture, Japan (40°53′N, 141°00′E) on 16 April 2003. Approximately 18 l of soil was collected from 15 sites (0–10 cm depth) at the nursery and was uniformly mixed. Table 1 shows the general characteristics of the Kanuma pumice and the nursery soil.

Fifty nonsterilized seeds were sown per plastic pot (90-mm height×100-mm diameter) that contained Kanuma pumice or nursery soil on 25 April 2003. Seeds were not covered with soil (Itoya and Shimoda 1995). Pots were kept in a greenhouse at the Tohoku Research Center, FFPRI, Morioka, Japan (39°46′N, 141°08′E). Twenty pots per soil were placed in a block, and 10 blocks were prepared for replications of each soil. After 1 mo, pots were transferred from the greenhouse to a nursery covered with plastic shading nets that allowed 32% of photosynthetically active radiation, as evaluated by using an LI-1400 datalogger

Table 1 General characteristics of Kanuma pumice and nursery soil (0–10 cm depth)

Characteristic	Kanuma Pumice	Nursery Soil
pH (KCl)	4.79±0.01	4.25±0.02***
Exchangeable Na (cg/kg)	22.9±0.90	22.3±1.09
Exchangeable K (cg/kg)	18.0±0.99	27.6±1.28**
Exchangeable Mg (cg/kg)	11.7±0.97	16.5±0.09**
Exchangeable Ca (cg/kg)	120.9±4.47	126.6±1.58
Available P (mg/kg)	Trace	22.2±2.21***
Total C (%)	0.13±0.01	9.34±0.07***
Total N (%)	0.01±0.00	0.56±0.00***
C/N	28.0±7.83	16.6±0.16

Results are expressed as value ±S.E. Differences between values for Kanuma pumice and nursery soil were evaluated by using Student's *t*–test.

***$P<0.001$, **$P<0.01$.

with a quantum sensor (Li-Cor, Lincoln, NE, USA). Seeds were watered every 2 or 3 d, and the position of the blocks was randomized every month. On 1 July 2003, five seedlings were randomly selected from pots of each soil type. Heights and basal diameters of the seedlings were measured before the collection. Seedling roots were rinsed with water and dried on filter paper. Fresh weights of the aboveground parts and roots of each seedling were measured separately (Table 2). Roots of these seedlings were used to isolate fungi on the root surfaces.

Fungal Isolation from Seeds Forty-five seeds were randomly selected and used to isolate fungi. Two seeds were placed separately per plate (90-mm diam) of potato dextrose broth (10 ml; Wako, Osaka, Japan) that contained 2% agar (PDA). They were incubated for 10 d in the dark at 25°C. Fungal colonies were detected with a microscope (×40 and ×100, Nikon Eclipse E400, Tokyo, Japan) and were inoculated onto acidic PDA (pH 3) that contained 0.025% citric acid to separate fungi from bacteria. The fungi were transferred again onto acidic PDA, cultivated for 7 d in the dark and then transferred and cultivated on normal PDA. Fungal genera were identified based on Watanabe (2002) and Barnett and Hunter (1998). Fungal detection frequency on the seeds was calculated as: FDF on seeds (%) = $(N_d/N_t) \times 100$, where N_d is the number of seeds from which the fungus was detected, and N_t is the total number of seeds (= 45).

Fungal Isolation from Soil Soil fungi were isolated by using the method of Warcup (1950). Four boxes (240×120×100 mm high) each were prepared to hold Kanuma pumice and nursery soil. Soil in each box was fully mixed, and water was added to the boxes until it poured from the bottom. The soil was incubated for 2 d at room temperature. The water contents of the Kanuma pumice and nursery soil after the incubation were 43.3% and 48.6%, respectively. Ten milligrams of Kanuma pumice were collected and crushed in sterile demineralized water (1 ml) by using sterile tweezers on a plastic plate (90-mm diam). Liquid PDA (10 ml, approximately 40°C) was poured into the plate and mixed fully with the crushed soil. The plate was incubated for 10 d in the dark at 25°C. Ten milligrams of nursery soil were collected and shaken with 10 ml of sterile water in a screw-topped test tube (120-mm height×25-mm diam) for 5 min under reciprocal rotation at 250 rpm. A 1-ml sample was then placed in a plastic plate (90-mm diam), and melted PDA (10 ml, approximately 40°C) was poured into the plate. The plate was incubated for 10 d in the dark at 25°C. Eight replications were prepared for each soil type. Fungal isolation methods and identification of genera were the same as for fungal isolation from seeds. The FDF in the soil was calculated as: FDF in soil (%) = $(N_d/N_t) \times 100$, where N_d is the number of plates from which the fungus was detected and N_t is the total number of plates (= 8).

Table 2 Fresh weight, height, and basal diameter of seedlings used for fungal isolation on the root surface

Seedlings	Total FW (mg ± SE)	FW of Leaves and Stem (mg ± SE)	FW of Roots (mg ± SE)	Height (mm ± SE)	Basal Diameter (mm ± SE)
Kanuma pumice	28.3±1.83	20.2±1.55	8.04±0.73	17.9±0.63	0.78±0.04
Nursery soil	34.4±3.10	25.6±1.75	8.76±1.61	19.6±1.06	0.88±0.07

No significant difference was detected between the soils for any variable (Student's *t*-test, $P>0.05$).

Fungal Isolation from Seedling Roots Mycelia were isolated from root surfaces of seedling growing in soils by using the method described by Harley and Waid (1955). Roots were rinsed twice with demineralized water and transferred to a screw-topped test tube (120-mm height×25-mm diam) that contained sterile demineralized water (7 ml). The test tube was reciprocally shaken at 250 rpm for 3 min, and then all the water was discarded, and sterile demineralized water (7 ml) was aseptically added to the test tube. The test tube was shaken again at 250 rpm for 3 min; this procedure was repeated nine times. Roots were then placed on a sterile filter paper (no. 1, Advantec, Tokyo, Japan) to remove extra water and were cut into 5-mm pieces on a glass plate. The pieces were separately placed onto PDA plates and incubated for 10 d in the dark at 25°C. Fungal isolation methods and identification of genera were the same as for fungal isolation from seeds. The FDF on roots was calculated as: FDF on roots (%) = $(N_d/N_t) \times 10$, where N_d is the number of root pieces from which the fungus was detected and N_t is the total number of root pieces. The FDF values for roots of five seedlings were averaged and used for correlation analyses.

Fungal Sensitivity to Seed Extract The following strains were isolated from seeds and soils and were used for the fungal sensitivity test: the seed fungi *Alternaria* (isolate nos. 1a, 26c), *Cladosporium* (isolate nos. 5a, 10e), *Pestalotiopsis* (isolate nos. 8b, 17b2, 35b), and *Phomopsis* (isolate nos. 3a, 6c); the Kanuma pumice fungi *Mortierella* (isolate nos. C, D) and *Mucor* (isolate nos. A, F); and the nursery soil fungi *Trichoderma* (isolate nos. 4-5, 3-10) and *Umbelopsis* (isolate nos. 9-3a, 10-3). These were the most frequently isolated genera. *Mucor* and the other fungal genera were identified to species level by rDNA sequence analysis by using primer pairs NL1/NL4 (O'Donnell 1993) and ITS1/ITS4 (White et al. 1990), respectively.

Soils (800 g) in 800 ml of demineralized water were autoclaved at 121°C for 20 min. The liquid was stored overnight at 4°C and filtered *in vacuo*. The filtrate was adjusted to 800 ml, 0.16 g of K_2HPO_4 and 1.6 g agar were added, and the mixture was autoclaved at 121°C for 20 min to produce Kanuma pumice extract agar (KPA) or nursery soil extract agar (NSA).

To produce seed extract, seeds (N=150, totaling 490 mg FW) were soaked in methanol (10 ml) and cut into pieces with a scalpel. The mixture was left for extraction overnight in the dark at room temperature. The extract was evaporated and stored at −20°C for later use.

The sensitivities of seed fungi and Kanuma pumice fungi were examined on KPA, and those of seed fungi and nursery soil fungi were examined on NSA. Methanol (50 μl) containing seed extract equivalent to 0.2, 0.4, or 0.8 seeds was added to a plastic plate (60-mm diam) and was mixed with melted KPA or NSA (5 ml, approximately 40°C). As a control, only methanol (50 μl) was used. A piece (2×2 mm) cut from the edge of freshly grown mycelia on PDA was inoculated at the center of a soil extract agar plate (60-mm diam). The plate was incubated in the dark at 25°C until the mycelial diameter on the control plate was approximately 30 mm for slow-growing fungi (*Alternaria*, *Cladosporium*) or approximately 40 mm for fast-growing fungi (*Pestalotiopsis*, *Phomopsis*, *Mortierella*, *Mucor*, *Umbelopsis*, *Trichoderma*); two mycelial diameters at right angles to each other were measured with a digital caliper square (Shinwa Rules Co., Ltd., Nigata, Japan). Four replications were prepared for each seed extract concentration, and the eight mycelial diameters were averaged.

The average mycelial growth on control plates was defined as 100%, and the relative growth rates on plates that contained seed extract were calculated. Regression analysis was performed to examine the relationship between the percentage of mycelial growth and seed extract concentration. All regression coefficients (P<0.05) were negative, so the absolute

value was used as an index of fungal sensitivity (Fig. 1). High fungal sensitivity values indicate that fungal growth was strongly inhibited by the seed extract. The fungal sensitivity values for the same species were averaged and used to calculate correlations with the FDF on root surfaces of the seedlings.

Antifungal Compounds of Seeds A thin-layer chromatography (TLC) bioautography assay was used to test the compounds in the seed extract against *Mucor* (strain A), which showed the highest sensitivity to the extract. Seed extract equivalent to 1.6 seeds was charged on a TLC plate (0.25 mm, Kieselgel 60 F_{254}, Merck, Darmstadt, Germany) and was developed in chloroform–methanol (9:1 *v*/*v*). A spore suspension in nutrient solution was sprayed over the developed TLC plate, which was then incubated at 25°C for 3 d (Homans and Fuchs 1970). Inhibitory zones were detected under UV light by binding with the fluorescent brightener Calcofluor White M2R (Woodward and Pearce 1985).

To isolate the compounds, 370 seeds (total=1.2 g FW) were soaked in methanol (20 ml) and cut into pieces with a scalpel, and the mixture was left for extraction overnight in the dark at room temperature. The extract (16.5 ml) was evaporated to produce an oily residue (246.5 mg), which was dissolved in ethyl acetate. The precipitate (45.8 mg) was removed on a filter paper, and the filtrate was evaporated to give an oily residue (220.6 mg). This residue (183.8 mg) was applied to a silica gel column (Wakogel C-60, 13 g, Wako, Tokyo, Japan) and eluted with chloroform–methanol (9:1). Fractions 13–17 (2 ml each) were concentrated together (90.6 mg). This residue (72.4 mg) was applied to a silica gel column (Wakogel C-60, 10 g) eluted with chloroform, and fractions 29–42 (1 ml each) were concentrated to 10.5 mg.

For identification, we used ^{1}H and ^{13}C NMR spectra and 2D NMR (H-H COSY, HMQC, HMBC, NOESY, and HOHAHA) with a JEOL Alpha-500 spectrometer (JEOL Ltd., Tokyo, Japan) to clarify the carbon sequences of the compound. The solvent was $CDCl_3$ and chemical shifts were recorded relative to the TMS (^{1}H) and solvent peaks (δ77.0°, ^{13}C). An EI–MS spectrum was obtained with a JMS-DX303HF mass spectrometer (JEOL Ltd., Tokyo, Japan) to clarify the fragmentation.

Quantification of Totarol Gas chromatography analysis was performed with an Auto System XL Gas Chromatograph (Perkin Elmer, Inc., Waltham, MA, USA) with a split-

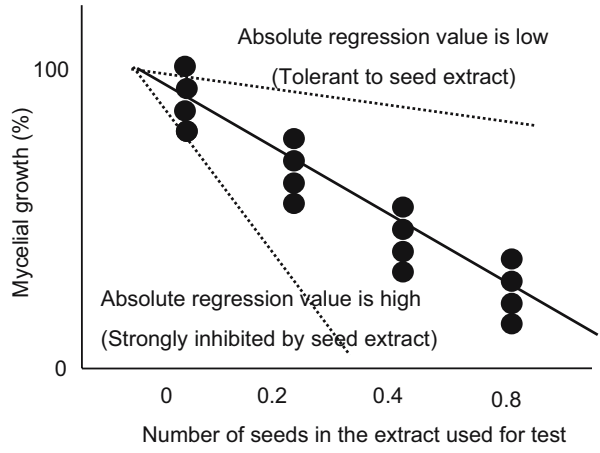


Fig. 1 Fungal sensitivity value. Regression analysis was performed between the percentage of mycelial growth and the concentration of the seed extract. All regression coefficients were negative ($P<0.05$), so the absolute value was used as an index of fungal sensitivity. High fungal sensitivity values indicate that fungal growth was strongly inhibited by the seed extract

splitless injector (1:10 ratio, 230°C) and FID detector (230°C). Nitrogen gas was used as a carrier gas at 1 ml min^{-1}. Hicap series CBP1 25 m×0.25 mm column (Shimadzu, Kyoto, Japan) was used for the analysis. The temperature program was 100–200°C at 10°C min^{-1}. The analysis was repeated three times, and the values were averaged. Standard curves were prepared as follows: totarol (0.138, 0.275, 0.55, 1.1 μg in 2 ml acetone; Sigma-Aldrich, St. Louis, MO, USA) was analyzed three times and values were averaged. The amount of totarol in the seed extract was read from the standard curve.

Examination of Fungal Sensitivity to Totarol Methanol (50 μl) that contained totarol equivalent to 0.2, 0.4, or 0.8 seeds (5.75, 11.5, and 23 μg, respectively) was added to a plastic plate (60-mm diam) and was mixed with KPA or NSA (5 ml, approximately 40°C). As a control, only methanol (50 μl) was used. Fungal inoculation and calculation of fungal sensitivity were the same as described for the fungal sensitivity to seed extract.

Statistical Analyses The StatView 5.0 (Macintosh) computer package (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. Fungal sensitivity was obtained by regression analysis. The relationship between FDF (%) on root surfaces and fungal sensitivity to seed extract or totarol were examined with Pearson's correlation test. Differences were considered significant at $P<0.05$.

Results

Seed Fungi Penicillium (several species; FDF 46.7%), *Alternaria* (40.0%), *Cladosporium* (35.6%), *Pestalotiopsis* (17.8%), and *Phomopsis* (8.9%) were the main isolates. *Alternaria* (isolate nos. 1a, 26c), *Cladosporium* (isolate nos. 5a, 10e), *Pestalotiopsis* (isolate nos. 8b, 17b2, 35b), and *Phomopsis* (isolate nos. 3a, 6c) were selected to examine fungal sensitivities to the seed extract and the antifungal compound totarol. *Papulospora* (FDF 6.7%), *Aspergillus* (2.2%), *Mortierella* (2.2%), *Phoma* (2.2%), *Sclerotium* (2.2%), and *Trichoderma* (2.2%) were also isolated.

Soil Fungi (Kanuma Pumice) Penicillium (several species; FDF 100.0%), *Mucor* (75.0%), *Cladosporium* (62.5%), *Mortierella* (50.0%), *Alternaria* (12.5%), *Aspergillus* (12.5%), *Aureobasidium* (12.5%), *Gliocladium* (12.5%), and *Ulocladium* (12.5%) were isolated. These *Cladosporium* and *Alternaria* species were different species from the seed fungi found in these genera. *Mortierella* (isolate nos. C, D) and *Mucor* (isolate nos. A, F) were selected to examine fungal sensitivities to the seed extract and totarol.

Soil Fungi (Nursery Soil) Penicillium (several species; FDF 100.0%), *Umbelopsis* (87.5%), *Trichoderma* (62.5%), *Truncatella* (50.0%), *Pestalotiopsis* (25.0%), *Mucor* (12.5%), and *Stagonospora* (12.5%) were isolated. The *Pestalotiopsis* species was different from the seed fungus in this genus. *Trichoderma* (isolate nos. 4-5, 3-10) and *Umbelopsis* (isolate nos. 9-3a, 10-3) were selected to examine fungal sensitivities to the seed extract and totarol.

Fungi on Roots (Kanuma Pumice) Penicillium (several species; FDF 38.4%), *Phomopsis* (24.4%), *Cladosporium* (21.8%), *Pestalotiopsis* (16.7%), *Alternaria* (15.6%), *Gliocladium* (11.3%), *Coniothyrium* (10.9%), *Staphylotrichum* (5.4%), and *Mortierella* (4.4%) were isolated. The *Mortierella* and *Gliocladium* species were the same as the Kanuma pumice

fungi in these genera. Several different *Penicillium* species originated from the seeds and soil.

Fungi on Roots (Nursery Soil) *Cladosporium* (FDF 26.0%), *Penicillium* (several species; 22.4%), *Trichoderma* (13.7%), *Phomopsis* (10.1%), *Pestalotiopsis* (9.0%), *Humicola* (6.2%), *Fusarium* (4.7%), *Alternaria* (4.2%), *Aspergillus* (4.2%), *Torula* (2.9%), *Papulaspora* (1.8%), *Geotrichum* (1.3%), and *Paecilomyces* (1.3%) were isolated. The *Alternaria*, *Cladosporium*, *Pestalotiopsis*, and *Phomopsis* species were the same as the seed fungi in these genera, and *Trichoderma* was the same species as the nursery soil fungus. Several different *Penicillium* originated from the seeds and soil.

Fungal Sensitivity to Seed Extract Fungal sensitivities to the seed extract on KPA were 45.6 for *Alternaria* (seed fungi), 25.0 for *Cladosporium* (seed fungi), 46.4 for *Phomopsis* (seed fungi), 45.6 for *Pestalotiopsis* (seed fungi), 80.0 for *Mortierella* (Kanuma pumice fungi), and 84.0 for *Mucor* (Kanuma pumice fungi). Fungal sensitivities to the seed extract on NSA were 57.5 for *Alternaria* (seed fungi), 22.1 for *Cladosporium* (seed fungi), 44.4 for *Pestalotiopsis* (seed fungi), 58.1 for *Phomopsis* (seed fungi), 41.4 for *Umbelopsis* (nursery soil fungi), and 38.2 for *Trichoderma* (nursery soil fungi).

Correlation Results Significant and negative correlations were found between the FDF on seedling root surfaces and fungal sensitivity to the seed extract in both soils ($P<0.05$; Fig. 2a,b). Correlation coefficients (r^2) for Kanuma pumice and nursery soil were −0.915 ($P=0.007$) and −0.834 ($P=0.038$), respectively.

Relationship between FDF on Root Surfaces and Fungal Sensitivity to Totarol Based on analyses of ^{1}H and ^{13}C NMR data and EI-MS data, we identified the antifungal compound in the seeds of *T. dolabrata* var. *hondai* as totarol (Fig. 3), which agreed with the NMR findings of Ying and Kubo (1991).

Fungal sensitivities to totarol on KPA were 68.6 for *Alternaria* (seed fungi), 55.1 for *Cladosporium* (seed fungi), 64.4 for *Phomopsis* (seed fungi), 62.2 for *Pestalotiopsis* (seed fungi), 75.2 for *Mortierella* (Kanuma pumice fungi), and 90.6 for *Mucor* (Kanuma pumice

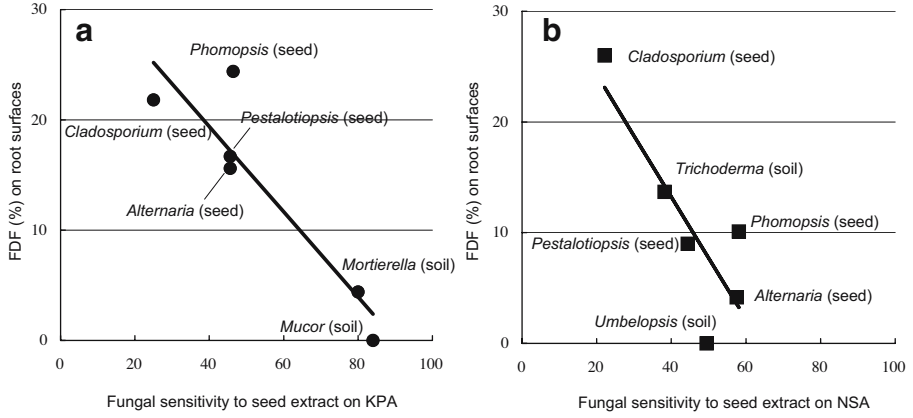


Fig. 2 Relationship between the fungal detection frequency (FDF) on the root surfaces and fungal sensitivity to seed extract: (**a**) Kanuma pumice ($r^2=-0.915$, $P=0.007$); (**b**) nursery soil ($r^2=-0.834$, $P=0.038$). The origin of each fungus is shown in parentheses

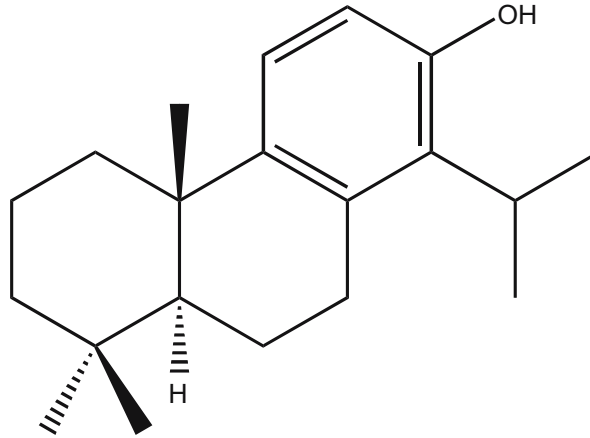


Fig. 3 Chemical structure of totarol

fungi); those on NSA were 70.2 for *Alternaria* (seed fungi), 40.5 for *Cladosporium* (seed fungi), 57.5 for *Pestalotiopsis* (seed fungi), 55.9 for *Phomopsis* (seed fungi), 56.7 for *Umbelopsis* (nursery soil fungi), and 54.1 for *Trichoderma* (nursery soil fungi).

Significant and negative correlations were found between the FDF on seedling root surfaces and fungal sensitivity to totarol in both soils ($P<0.05$; Fig. 4a,b). Correlation coefficients for Kanuma pumice and nursery soil were −0.898 ($P=0.01$) and −0.814 ($P=0.049$), respectively.

Discussion

The FDF values on root surfaces in both soils were significantly correlated with the fungal sensitivity to the seed extract and totarol, an antifungal compound of seeds. Our results indicate that seed and soil fungi tolerant of totarol can grow on the root surfaces of *T.*

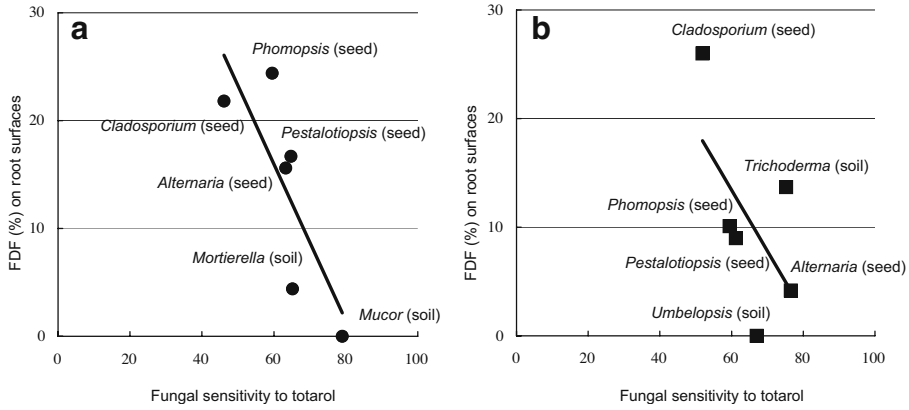


Fig. 4 Relationship between the fungal detection frequency (FDF) on the root surfaces and fungal sensitivity to totarol. (**a**) Kanuma pumice ($r^2=-0.898$, $P=0.01$). (**b**) Nursery soil ($r^2=-0.814$, $P=0.049$). The origin of each fungus is shown in parentheses

dolabrata var. *hondai* seedlings. Totarol has been isolated from *T. dolabrata* var. *hondai* seeds (this study; Hasegawa and Hirose 1982) as well as from *Pilgerodendron uviferum* (D. Don) Florin bark extracts, from which it was shown to be antifungal against *Rhizoctonia solani* and *Phragmidium violaceum* (Solís et al. 2004). In our study, totarol was also detected in Kanuma pumice and nursery soil (0.38±0.08 μg g^{-1} soil FW and 0.61±0.16 μg g^{-1} soil FW, respectively) in which 2-mo-old *T. dolabrata* var. *hondai* seedlings were growing. The seedling roots also contained totarol (0.45±0.06 μg g^{-1} root FW grown in Kanuma pumice and 0.84±0.16 μg g^{-1} root FW grown in nursery soil, respectively), and thus, totarol would select fungi on root surfaces of the seedlings in the early growth stage. α- and β-Eudesmol have been isolated from *T. dolabrata* var. *dolabrata* seeds (Hasegawa and Hirose 1982). β-Eudesmol has been extracted from amboyna wood and is antifungal against the wood-rotting fungus *Pleurotus pulmonarius* (Kusuma et al. 2004). In our study, mixtures of α-, β-, and γ-eudesmol (2:2:1) were isolated from *T. dolabrata* var. *hondai* seeds; these compounds were identified by analyses of ^{1}H and ^{13}C NMR data, which agreed fully with those of Raharivelomanana and Bianchini (1995). However, the amount of eudesmols in the seeds was too low to be antifungal against the tested fungi, except for *Mucor* (4.95 μg per seed). Therefore, we conclude that totarol is the main antifungal compound in the *T. dolabrata* var. *hondai* seeds.

Soil nutrient conditions influence mycelial growth, and the rate of mycelial growth seems to be one factor that selects fungi in the rhizosphere. We also examined the rates of mycelial growth of seed and soil fungi on both KPA and NSA. However, the mycelial growth rates were not related to the FDF on the roots (r^2=−0.11, P=0.85 for Kanuma pumice; r^2=0.05, P=0.93 for nursery soil). This result indicates that the nutrient differences between Kanuma pumice and nursery soil did not affect which fungi grew on the seedling root surfaces.

The seed extract would include amino acids and vitamins that stimulate fungal growth. Therefore, the correlation between FDF and fungal sensitivity to the seed extract was different from that between FDF and fungal sensitivity to totarol (Figs. 2, 4). Complicated interactions between chemical compounds (including antifungal compounds and nutrients) and fungal growth would be expected.

Our method (Fig. 1) did not allow us to compare fungal sensitivity values with the minimum inhibitory concentration (MIC). To examine the interaction between an antifungal compound and fungal growth in a natural environment, fungal sensitivity values need to be better than MIC, which is calculated by using an impractically high amount of antifungal compounds against tolerant fungi. Because our fungal sensitivity values reflect the actual amount of antifungal compounds in plant tissues, these values should more accurately estimate the influence of antifungal compound on fungi.

Seeds were collected directly from felled *T. dolabrata* var. *hondai* trees, and thus, seed fungi would be epiphytic on trees. *Alternaria*, *Cladosporium*, *Penicillium*, and *Trichoderma* have been isolated from various conifer seeds (Watanabe 2002), and thus are not specific to *T. dolabrata* var. *hondai* seeds. *Pestalotiopsis* has weak pathogenicity to conifer seedlings, and *Phomopsis* causes cankers of conifer trees and seedlings (Ito 1974). Future work should examine the influences of these fungi on *T. dolabrata* var. *hondai* seedlings.

Based on the findings of this study, we conclude that the antifungal compound totarol that is produced by *T. dolabrata* var. *hondai* seeds is one factor in the selection of fungi on root surfaces in the early growth stage.

Acknowledgments We thank the Hiranai Hiba Forestry Research Group for providing us with nursery soil; the Forestry Technology Center, Tohoku Regional Forest Office, for helping with the seed collection; and Mr.

N. Shimoda for technical help with our work in the nursery. Financial support was provided in part by Research Fellowships of Japan Society for the Promotion of Science for Young Scientists and by a research grant of the Forestry and Forest Products Research Institute, Japan.

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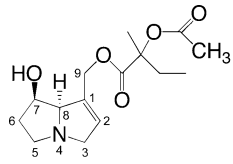
1999; Hartmann and Ober 2000; Eisner et al. 2002; Pasteels et al. 2003; Conner and Weller 2004). Many aspects remain to be explored and can be expected to reveal rich and rewarding examples of evolutionary biology.

PAs taken up from host plants are often converted into insect-PAs in which the acid moiety of plant origin is replaced by small, branched, aliphatic 2-hydroxy acids of insect origin. Commonly found insect-PAs include callimorphine (**1**), the 2-acetoxy-2-methylbutanoic O^9 ester of retronecine (**2**), creatonotine A, 9-(2-hydroxy-3-methylbutanoyl) retronecine, and creatonotine B (**3**), 9-(2-hydroxy-3-methylpentanoyl) retronecine (Fig. 1; Aplin et al. 1968; Edgar et al. 1980; Hartmann et al. 1990, 2004a; Schulz et al. 1993). Insect-PAs are predominantly esters of **2**. Insects that ingest PAs with a heliotridine (**4**) moiety commonly invert the C^7 (*S*) hydroxyl to C^7 (*R*); for example, 7*S*-heliotrine (**5**) is converted to 7*R*-*epi*-heliotrine (**6**) by several species before formation of insect-PAs (Wink et al. 1988; Schulz et al. 1993). Insect-PAs with a retronecine (**2**) moiety also occur as O^7 esters including isocallimorphine (**7**) and isocreatonotine B (**8**) (Hartmann et al. 1990, 2004a).

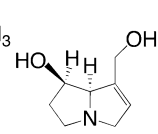
Hartmann et al. (2003b, 2004a, b, 2005a) have presented evidence that insect-PAs, specifically creatonotines, are converted into the pheromone hydroxydanaidal (**9**) in males of the arctiid moth *Estigmene acrea*, but no biosynthetic reason has been given for why insect-PAs are involved, and it is not immediately clear why this would be the case from consideration of the currently accepted biosynthetic pathway for hydroxydanaidal (**9**) proposed by Schulz et al. (1993) (Scheme 1, dotted arrows). It also is not known if insect-PAs are the precursors of **9** in other species. We have, therefore, looked for evidence that insect-PAs are also involved in the biosynthesis of hydroxydanaidal (**9**) in the arctiid moth *Creatonotos transiens* and have considered possible mechanisms of biosynthesis of hydroxydanaidal (**9**) that specifically require insect-PAs as precursors. In a preliminary investigation, we established that when final male instars of *C. transiens* are fed with pure supinine (**10**) (an alkaloid lacking a C^7 hydroxyl), they produce the insect-PAs 7-desoxycallimorphine (**11**) and estigmine B (=7-desoxycreatonotine B) (**12**), which do not proceed to hydroxydanaidal (**9**) or produce its 7-desoxy analogue danaidal in the adult moths. Thus, **10** can be used as an indicator of insect-PA biosynthesis activity. We have, thus, used **10** to indicate insect-PA biosynthetic activity in *C. transiens*, while simultaneously feeding increasing amounts of **5**, a PA known to produce **9,** to look for evidence that insect-PAs derived from **5** are intermediates in the biosynthesis of **9** in *C. transiens*.

Methods and Materials

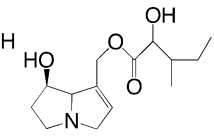
GC-MS and LC-MS Analysis Electron impact mass spectra (70 eV) were obtained with a Hewlett-Packard 5972 mass spectrometer coupled to a Hewlett-Packard 5890 series II gas chromatograph equipped with splitless on-column injection and an SGE BPX5 column: 30 m×0.25 mm 0.5 μm film thickness. The injector and transfer line were set at 250°C, ion source temperature was 150°C, and gas chromatograph oven temperature was 80°C for 2 min then programmed to 300°C at 20°C/min. The carrier gas was helium at 1.6 ml/min and spectra were recorded at 70 eV. Liquid chromatography/mass spectrometry (LC-ESI-MS) was performed by using a ThermoFinnigan Surveyor autosampler and liquid chromatography system coupled to a ThermoFinnigan LCQ ion-trap mass spectrometer as described previously by Boppré et al. (2005).



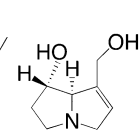
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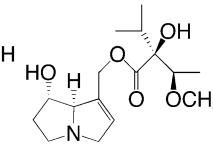
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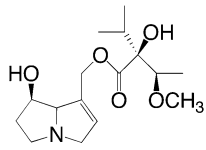
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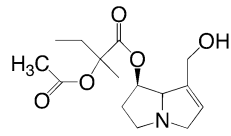
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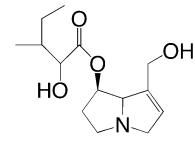
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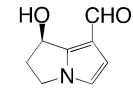
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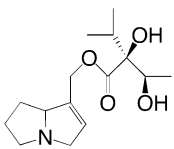
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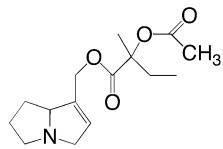
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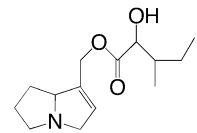
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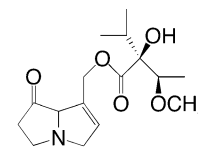
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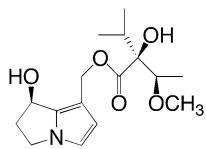
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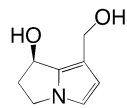
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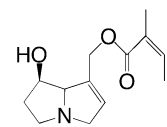
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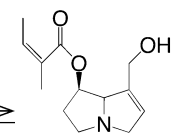
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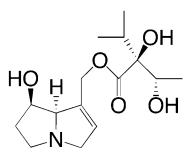
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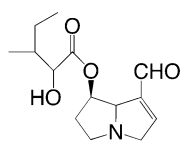
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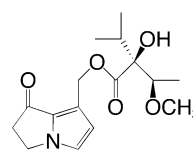
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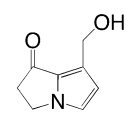
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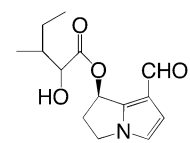
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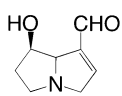
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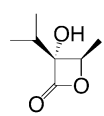
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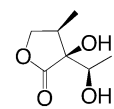
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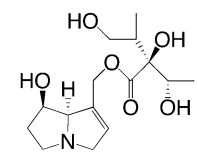
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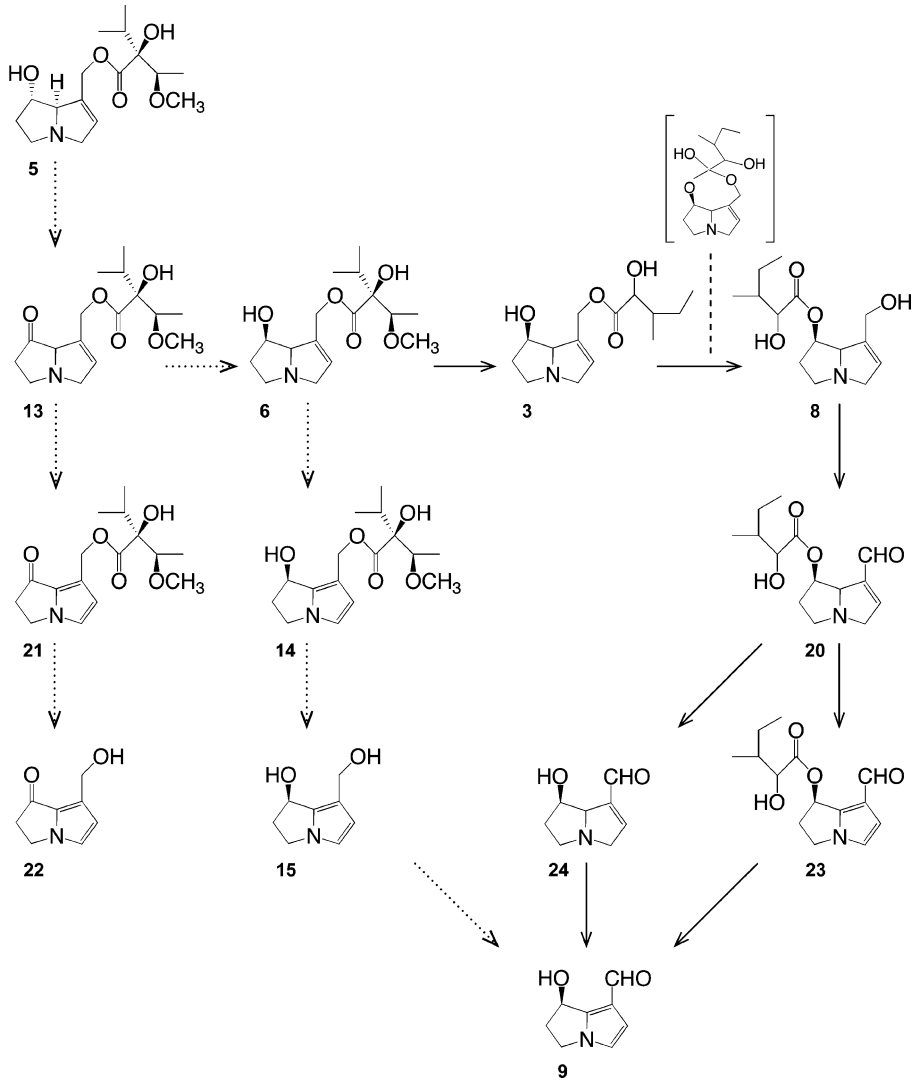
Fig. 1 Structures of some 1,2-dehydro-pyrrolizidine ester alkaloids (PAs), insect-PAs, and dihydropyrrolizines, including insect pheromones, derived from these. For simplicity, the PAs shown are depicted as free bases, although they usually occur and are strictly maintained in plants and insects as *N*-oxides (see review by Hartmann and Ober 2000). It should be noted, however, that the dihydropyrrolizine ("pyrrole") oxidation products shown, viz. **9**, **14**, **15**, **21**, **22**, and **23**, do not occur as *N*-oxides because the nitrogen lone pair electrons required for *N*-oxidation are delocalized in the aromatic "pyrrole" ring system. Callimorphine (**1**), retronecine (**2**), creatonotine B (**3**), heliotridine (**4**), heliotrine (**5**), 7*R*-*epi*-heliotrine (**6**), isocallimorphine (**7**), isocreatonotine B (**8**), 7*R*-hydroxydanaidal (**9**), supinine (**10**), desoxycallimorphine (**11**), estigmine B (=7-desoxycreatonotine B) (**12**), 7-ketoheliotrine (**13**), dehydro-7*R*-*epi*-heliotrine (**14**), dehydro-retronecine (**15**), anhydro-retronecine (**16**), 9-angeloylretronecine (**17**), 7-angeloylretronecine (**18**), lycopsamine (**19**), isocreatonotine B aldehyde (**20**), dehydro-7-ketoheliotrine (**21**), loroquin (**22**), dehydro-isocreatonotine B aldehyde (**23**), retronecine aldehyde (**24**), viridiflorine β-lactone (**25**), ithomiolide A (**26**), and hydroxy-lycopsamine (**27**)

Thin Layer Chromatography Thin layer chromatography (TLC) was performed on Polygram® SIL G/UV254 precoated plastic sheets (Merck) with chloroform/methanol/conc. ammonia (85:14.5:0.5) as solvent. PAs were made visible by iodine vapor or by spraying with chloranil and subsequently Ehrlich reagent (Molyneux and Roitman 1980).

Chemicals Pure hydroxydanaidal (**9**), supinine (**10**), and heliotrine (**5**) were obtained from a collection of PAs, and their metabolites held at CSIRO Australian Animal Health Laboratory, Geelong, Australia, where they were isolated from various plant sources or synthesized and characterized by nuclear magnetic resonance, mass spectra, microanalysis, melting point, and comparison with authentic samples.

Identification of PAs and Their Metabolites PAs **10** and **5**, extracted from *C. transiens*, were identified by comparison of their TLC R_f values, GC retention times (13.42 and 14.25, respectively), mass spectra molecular weights (*m*/*z* 283 and 313, respectively), and MS fragmentation patterns with those of the authentic standards given to the larvae. Hydroxydanaidal (**9**) was identified also by comparison of TLC R_f and GC retention time (10.01 min) and mass spectrum (M^+, *m*/*z* 151) with that of an authentic standard. The insect-PAs callimorphine (**1**) (GC retention time 13.35 min; M^+, *m*/*z* 297), creatonotine B (**3**) (13.65 min; M^+, *m*/*z* 269), isocreatonotine B (**8**) (13.46 min; M^+, *m*/*z* 269), 7-desoxycallimorphine (**11**) (12.44 min; M^+, *m*/*z* 281), estigmine B (=7-desoxycreatonotine B) (**12**) (12.56 min; M^+, *m*/*z* 253), and 7*R*-*epi*-heliotrine (**6**) (14.15 min; M^+, *m*/*z* 313) were tentatively identified by comparison of their electron impact spectra with published spectra (Edgar et al. 1980; Wink et al. 1988; Ehmke et al. 1990; Hartmann et al. 2004a). Molecular weights were confirmed by LC-ESI-MS.

Feeding Experiments Larvae of *C. transiens* (Walker), from an established laboratory culture that originated from females collected in Indonesia and maintained on an artificial diet (Bergomaz and Boppré 1986), were used. Four groups of three to five male and four groups of three to five female larvae were each given either 500 μg supinine (**10**) alone (group A); or 500 μg **10** plus 200 μg **5**, (group B); 500 μg **10** plus 500 μg **5**, (group C); or 500 μg **10** plus 1,000 μg **5** (group D). The alkaloid stock solutions were made up in reagent grade methanol, and the appropriate volumes were applied to glass microfiber filter discs (Whatman® GF/B, 2.1 cm), which were offered to final instars as their first meal after molting. After the larvae had consumed the entire sample, they were fed with PA-free artificial diet until pupation [larvae of *Creatonotos* are pharmacophagous with respect to PAs—thus, pure PAs act as feeding stimulants, and larvae consume PAs offered on glass



Scheme 1 Showing the previous (*dotted arrows*; Schulz et al. 1993) and proposed (*solid arrows*) mechanisms for conversion of **5** to **9**. For simplicity, PAs are shown as free bases, although in the insects, they are strictly maintained as *N*-oxides (Hartmann and Ober 2000). It should be noted, however, that, as indicated in the legend of Fig. 1, the non-alkaloidal dihydropyrrolizine oxidation products, viz. **9**, **14**, **15**, **21**, **22**, and **23** cannot form *N*-oxides. Creatonotine B (**3**), heliotrine (**5**), 7*R*-*epi*-heliotrine (**6**), isocreatonotine B (**8**), 7*R*-hydroxydanaidal (**9**), 7-ketoheliotrine (**13**), dehydro-7*R*-*epi*-heliotrine (**14**), dehydro-retronecine (**15**), isocreatonotine B aldehyde (**20**), dehydro-7-ketoheliotrine (**21**), loroquin (**22**), dehydro-isocreatonotine B aldehyde (**23**), retronecine aldehyde (**24**)

microfiber (or other materials), thus allowing for quantiative feeding with PAs; cf. Boppré and Schneider 1985].

Quantification of PAs Newly emerged adults were held for 24 hr before the male coremata were artificially protruded and excised. The moths from each group were combined and macerated in methanol, filtered, and the methanol evaporated. The residue was dissolved in 2 N H_2SO_4, treated with Zn dust, and stirred at room temperature for 3 hr before being filtered. The filtrate was extracted three times with ether. The acid solution was then basified to above pH 10 with conc. ammonium hydroxide and extracted three times with an equal volume of dichloromethane. The dichloromethane was dried with anhydrous sodium sulphate, filtered, evaporated, and the residue transferred into a vial and dissolved in methanol ready for gas chromatography–mass spectrometry (GC-MS), LC-ESI-MS, and TLC analysis.

Adult moths reared on PA-free diet were injected with each of the stock solutions of the various mixtures of **10** and **5** used in the feeding experiments, and then extracted by the standard procedure to provide recovery data for GC-MS comparison with levels found in moths fed with these solutions as larvae.

Coremata from males were extracted with dichloromethane. Aliquots were immediately analyzed by GC-MS and TLC. The amount of **9** was estimated by GC-MS comparison with an authentic standard.

Results

Female *C. transiens* moths given only 500 μg of supinine (**10**) as final instars stored 260 μg (52%) in their bodies (Fig. 2a, ♀; Table 1). Only a trace of the insect-PA **11** was detected (Fig. 2a, ♀; Table 1). A similar level of storage of **10**, accompanied by traces of **11**, persisted as the amount of heliotrine (**5**) increased in the larval diet, and no insect-PAs derived from **5** were detected (Fig. 2b–d, ♀; Table 1). The virtual absence of insect-PAs in all groups indicates that female *C. transiens* have a limited capability for producing insect-PAs. A significant quantity of **5** fed to the female larvae was stored in the adult bodies as approximately equal amounts of **5** and 7*R-epi*-heliotrine (**6**) (Fig. 2b–d, ♀; Table 1), indicating that the females also have a limited ability to epimerise the C^7 hydroxyl group of **5**.

In marked contrast to females, male *C. transiens* given only 500 μg of pure **10** converted 318 μg to insect-PAs **11** and **12** (Table 2; Fig. 2a, ♂). Only a small amount (60 μg) was stored as unchanged **10** (Fig. 2a, ♂). No hydroxydanaidal (**9**) (Table 2) or danaidal, the C^7 desoxy analogue of **9**, was detected on the enlarged coremata. When 200 μg of **5** was fed along with 500 μg of **10** (Fig. 2b, ♂), a high proportion of the former (approximately 50%), allowing for molecular weight differences, 151 (**9**) vs 313 (**5**), was converted to **9**, also on enlarged coremata (Table 2). There was no evidence of **5** in the body extracts (Fig. 3b, ♂), and only a small amount (7 μg) of 7*R-epi*-heliotrine (**6**) (Table 2, Fig. 2b, ♂). No insect-PAs from **5** or **6** were detected, but the 7-desoxy analogues **11** and **12** from **10** were, as before (Fig. 2a, ♂), present in high amounts (Table 2, Fig. 2b, ♂). The insect-PAs **11** and **12** were, respectively, 39 and 36% by weight of the PAs detected in the extract of the bodies, excluding the pheromone **9** (Fig. 2b, ♂). Compounds **10** (23%) and **6** (2%) made up the remainder of the PAs in the body extract. The absence of insect-PAs derived from **5** and **6**

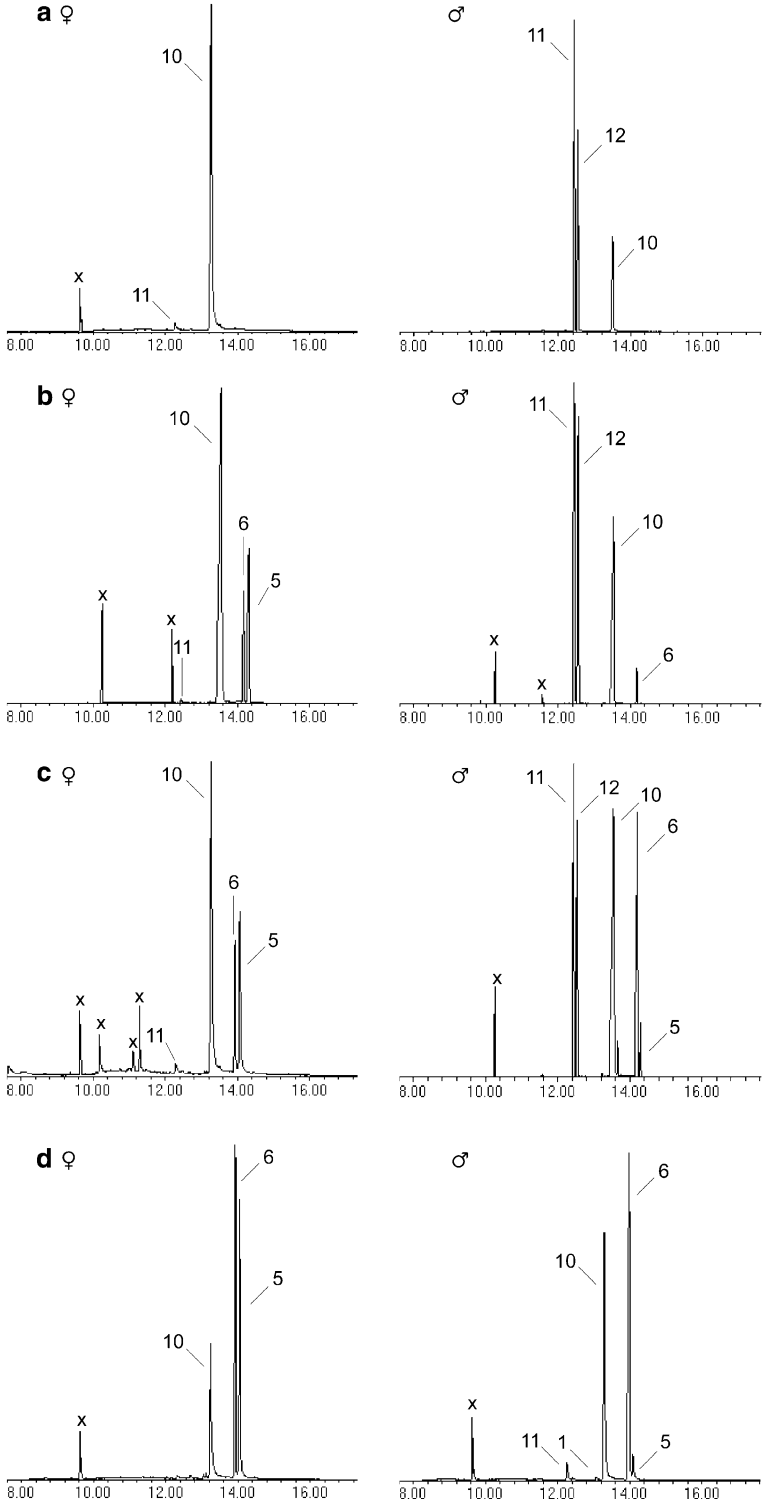

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Fig. 2 GC-MS total ion chromatograms of alkaloid extracts of female and male *C. transiens* moths given in final larval stage 500 μg **10** (**a**), 500 μg **10** plus 200 μg **5** (**b**), 500 μg **10** plus 500 μg **5** (**c**), and 500 μg **10** plus 1,000 μg **5** (**d**). *x*=contaminant. For structures, see Fig. 1

but high levels of **11** and **12** that arise from **10** supports the hypothesis that insect-PAs originating from **5** and **6** were being used to produce **9**.

When the male *C. transiens* larvae were given a mixture of 500 μg of supinine (**10**) and 500 μg of heliotrine (**5**), a small amount of **5** (9 μg, 2% of total PAs in the bodies) was seen in the adults, along with an increased amount (50 μg, 12%) of 7*R-epi*-heliotrine (**6**) (Fig. 2c, ♂, Table 2). Only traces of insect-PAs from **6** were present, in agreement with the idea that they are intermediates in the formation of **9**, and were being rapidly used for that purpose. About 75 μg of **9**, equivalent to 30% of the amount of **5** fed, were detected on the enlarged coremata (Table 2). Evidence of significant insect-PA formation activity was again indicated by the presence of high levels of the 7-desoxy insect-PAs, **11** (33%) and **12** (26%), derived from supinine (**10**). Unchanged **10** represented 27% of the total PAs in the bodies (Fig. 2c, ♂, Table 2).

In the last male group, in which larvae received 1,000 μg of **5** and 500 μg of **10**, we believe that the requirement for **9** is largely satisfied, and the profile of alkaloids in the bodies (Fig. 2d, ♂) was similar to that seen in the equivalent female group (Fig. 2d, ♀) except that females were less efficient in converting **5** to **6**. Compound **6** represented 32% by weight of PAs present in the body extract of the males, and **10** was 62% of the stored PAs (Fig. 2d, ♂), the reverse of that seen in group D females. A small amount of the insect-PA **11** was present, and a trace of **1** was detected (Fig. 2d, ♂). As in the females, there were barely detectable levels of insect-PAs from either **10** or **5** present in males. The total ion chromatogram shown in Fig. 2d, ♂ was also, in respect to **5** and its metabolites, similar to that seen when 1,000 μg of **5** alone were fed to final instar males (Edgar et al., unpublished data). The virtual absence of insect-PAs, even those arising from **10** when 1,000 μg of heliotrine (**5**) are given to final instar male *C. transiens* larvae, is noteworthy. It suggests that **5**, or more specifically its epimer **6**, became the primary substrate for insect-PA production so that no insect-PAs from **10** were generated in males, and all of the insect-PAs from **6** were converted to **9**. An estimated 287 μg per moth of hydroxydanaidal (**9**) were

Table 1 PAs and insect-PAs found per individual in the bodies of female *Creatonotos transiens* moths fed mixtures of supinine (**10**) and heliotrine (**5**) (groups A♀–D♀) as final instars

♂ Group	Fed		Recovered Supinine (**10**) and **11**			Recovered Heliotrine (**5**) and **6**		
	10 (μg)	**5** (μg)	Total (μg; abs. %)	**10** (μg; rel. %)[a]	**11** (μg; rel. %)[a]	Total (μg; abs. %)	**5** (μg; rel. %)[a]	**6** (μg; rel. %)[a]
A	500	–	260 (52)	260 (100)	Trace	–	–	–
B	500	200	373 (75)	373 (100)	Trace	143 (72)	83 (58)	60 (42)
C	500	500	387 (77)	387 (100)	Trace	165 (33)	90 (55)	75 (45)
D	500	1,000	280 (56)	280 (100)	–	453 (45)	213 (47)	240 (53)

The only insect-PA formed, in trace quantity, from **10** was 7-desoxycallimorphine (**11**). **5** was partially epimerized to 7*R-epi*-heliotrine (**6**)

[a] Percentage of recovered compounds

Table 2 PAs and insect-PAs found per individual in the bodies of male *Creatonotos transiens* moths fed mixtures of supinine (**10**) and heliotrine (**5**) (groups A♂–D♂) as final instars

♂ Group	Fed		Recovered Supinine (**10**) and Derivatives				Recovered Heliotrine (**5**) and Derivatives			
	10 (μg)	**5** (μg)	Total (μg; abs. %)	**10** (μg; rel. %)[a]	**11** (μg; rel. %)[a]	**12** (μg; rel. %)[a]	Total (μg; abs. %)	**5** (μg; rel. %)[a]	**6** (μg; rel. %)[a]	**9** (μg; rel. %)[a]
A	500	–	378 (77)	60 (16)	193 (51)	125 (33)	–	–	–	–
B	500	200	427 (85)	102 (24)	170 (40)	155 (36)	109 (54)[b]	–	7 (6)	51 (94[b])
C	500	500	380 (76)	120 (32)	145 (38)	115 (30)	209 (42)[b]	9 (4)	50 (24)	75 (72[b])
D	500	1,000	393 (78)	372 (95)	21 (5)	–	767 (77)[b]	12 (2)	191 (25)	282 (73[b])

Insect-PAs formed from **10** were 7-desoxycallimorphine (**11**) and 7-desoxycreatonotine B (**12**). **5** was completely or partially epimerized to 7*R-epi*-heliotrine (**6**) then partially converted, via insect-PAs, e.g., creatonotine B (**3**) (only trace amounts of which were seen, Fig. 3, ♂), into hydroxydanaidal (**9**), which was secreted on coremata as a pheromone

[a] Percentage of recovered compounds

[b] Values for **9** are doubled to compensate for molecular weight differences between **9** and **5** (151 vs 313)

detected on enlarged coremata (Table 2), and this represented 57% of the heliotrine (**5**) fed to the larvae.

Discussion

*Insect-PAs as Precursors of Hydroxydanaidal (**9**) in C. transiens* The data obtained from feeding mixtures of **10** and **5** to final instars of *C. transiens* strongly support the hypothesis that insect-PAs that arise from **5** are intermediates in the biosynthesis of the pheromone hydroxydanaidal (**9**) in male *C. transiens*, in agreement with a similar conclusion drawn by Hartmann et al. (2003b, 2004a, b) in regard to formation of **9** in male *E. acrea*. By using supinine (**10**) as an indicator of insect-PA biosynthetic activity and heliotrine (**5**) as a precursor of hydroxydanaidal (**9**), it was apparent that insect-PAs formed from **5** were being rapidly and almost completely converted into hydroxydanaidal (**9**) in male *C. transiens*. The fact that female *C. transiens* were significantly less efficient in epimerizing the 7*S*-hydroxyl and in producing insect-PAs also points to the insect-PAs in *C. transiens* being primarily required to produce the male pheromone hydroxydanaidal (**9**).

*Mechanism for Formation of Hydroxydanaidal (**9**) from Insect-PAs* Heliotrine (**5**) is a well-established precursor of 7*R*-hydroxydanaidal (**9**) in *C. transiens* (Bell et al. 1984; Wink et al. 1988; Schulz et al. 1993). Schulz et al. (1993) proposed the currently accepted biosynthetic pathway from **5** to **9** (Scheme 1, dotted arrows). They demonstrated that *C. transiens* first oxidizes the 7*S*-hydroxyl of **5** to give 7-ketoheliotrine (**13**), which is then stereo-selectively reduced to 7*R-epi*-heliotrine (**6**), the epimer with the correct stereochemistry for **9**. They proposed that **6** is then aromatized to dehydro-7*R-epi*-heliotrine (**14**), hydrolyzed to dehydro-retronecine (**15**), and finally the primary hydroxyl is oxidized to give **9**. This proposal has been widely accepted as a model for the biosynthesis of **9** from PAs by insects, but it was put forward before it was found that insect-PAs, e.g., creatonotine B (**3**), may be required as intermediates in formation of **9** (Hartmann et al. 2003b, 2005b). The pathway proposed by Schulz et al. (1993) also involves formation of chemically reactive, hazardous dihydropyrrolizine ("pyrrole") intermediates such as **14** and **15**. These

are the very PA metabolites responsible for PA toxicity that PA-adapted insects, such as *C. transiens*, have evolved a specific mechanism to avoid, by maintaining PAs as non-toxic *N*-oxides that cannot be oxidized by cytochrome P450 isozymes to pyrrole esters such as **14** (Hartmann 1999). The mechanism of toxicity of 1,2-dehydropyrrolizidine ester alkaloid free bases, e.g., **6**, requires their metabolic conversion, *in vivo*, by cytochrome P450 enyzmes into dihydropyrrolizines, e.g., **14**, that are highly reactive biological alkylating agents. The relatively inert C^9 ester and C^7 alcohol or ester groups of alkaloids, such as **6,** become activated benzylic esters and alcohols once converted to dihydropyrrolizines, such as **14**. Compound **14** and its hydrolysis product **15** spontaneously generate carbonium ions at C^7 and C^9, and these immediately react with, and cross link, DNA and proteins at the site of their formation *in vivo*. This results in damage to biological systems in the organism unfortunate enough to consume PAs of this type. P450 enzymes are widespread, and they play important general and specific roles in virtually all organisms, including insects (Werck-Reichhart and Feyereisen 2000), so that 1,2-dehydro ester PAs can be expected to have broad-ranging toxicity against a wide spectrum of organisms, including insects. Abundant generalist insect herbivores avoid PA plants, indicating the anti-feeding and/or toxicity of PAs. Those insects that have adapted to PAs must actively maintain them as *N*-oxides that, unlike the free bases, are not oxidized to dihydropyrrolizines such as **14** by P450 enzymes. Thus, doubts are raised about the biosynthetic pathway suggested by Schulz et al. (1993), both by the recently demonstrated involvement of insect-PAs in the biosynthesis of **9** (Hartmann et al. 2003b) and the hazardous nature of the proposed chemically reactive dihydropyrrolizine ester intermediates (e.g., **14**).

The question arises: What are the structural features or chemical properties that make insect-PAs, such as **3**, more suitable for conversion into **9** than superficially similar alkaloids of plant origin such as **6**? We propose a biosynthetic pathway (Scheme 1, solid arrows) that specifically requires the structural attributes of insect-PAs, i.e., 7*R* stereochemistry and low molecular weight esterifying acids. This pathway, illustrated with creatonotine B (**3**), is equally applicable to other known retronecine ester insect-PAs, e.g., callimorphine (**1**), although Hartmann et al. (2005b) have concluded that the creatonotines, e.g., **3**, rather than callimorphines, e.g., **1**, are the specific precursors of **9** in *E. acrea*. Our proposal avoids the intermediacy of hazardous dihydropyrrolizine esters such as **14** or their hydrolysis product **15**. A key step is the intramolecular transesterification of O^9 ester insect-PAs, e.g., **3**, formed from 7*R*-*epi*-heliotrine (**6**), to the O^7 ester insect-PAs, e.g., **8**. This is only possible in the case of retronecine (**2**) esters with low molecular weight esterifying acids.

In retronecine (**2**), unlike heliotridine (**4**), the hydroxyl groups at C^9 and C^7 are close together, thus enabling, for example, the formation of the ether anhydro-retronecine (**16**) (Bull et al. 1968). Formation of such an ether structure is not physically possible with **4** where the hydroxyls are widely separated (Giordan et al., 1996). More importantly in the current context is the ability we have observed of retronecine O^9 monoesters such as 9-angeloylretronecine (**17**) to undergo intramolecular transesterification to 7-angeloylretronecine (**18**) in conc. aqueous NH_4OH at room temperature (unpublished data). The insect-PAs occur as both O^9 and O^7 esters, e.g., **1** and **7** and **3** and **8**, indicating that intramolecular O^9–O^7 transesterification is occurring with these molecules *in vivo*. Further evidence for this comes from the observation that rare insect-PAs with a heliotridine (**4**) moiety, which are unable to undergo O^9–O^7 intramolecular transesterification, have so far only been reported as O^9 esters (Hartmann et al. 2005b).

We propose (Scheme 1, solid arrows) that retronecine-based O^9 ester insect-PAs, e.g., **3**, are converted to O^7 ester insect-PAs, e.g., **8**, by internal, intramolecular transesterification.

If the insect-PAs were formed directly as O^7 esters, no intramolecular transesterification would be required. Intramolecular transesterification does not occur with any retronecine (**2**)-based PA monoesters. Bulky acids, for example, heliotric or viridifloric acids found in 7*R-epi*-heliotrine (**6**) and lycopsamine (**19**), appear not to be capable of intramolecular transesterification, and the O^7 ester isomers of **6** and **19** have never been found in nature. We note, however, that (Hartmann et al.2003a) have found the O^7 ester, 5′-demethylisolycopsamine (=isoideamine A), in extracts of the chrysomelid beetle *Platyphora boucardi*. They attribute this to intramolecular transesterification of the corresponding O^9 ester (ideamine A), suggesting that the acid involved, 2,3-dihydroxy-2-ethylbutanoic acid, may be near the upper size limit of acids able to undergo intramolecular O^9–O^7 transesterification and that the extra methyl group(s) present in heliotric and viridifloric acid, the esterifying acids of **6** and **19**, may be sufficient to prevent intramolecular O^9–O^7 transesterification. Brehm et al. (2007) have also found ideamine A and isoideamine A in *Prestonia amabilis* (Apocynaceae). Most PAs present in arctiine larval host plants have much bulkier O^9 esterifying acids than those found in insect-PAs and are unlikely to undergo O^9–O^7 intramolecular transesterification and, thus, according to our proposed pathway, cannot be used by *C. transiens* to make hydroxydanaidal (**9**). We, therefore, propose that *C. transiens* must convert some of the more complex plant PAs they ingest with their larval host plants into insect-PAs for intramolecular O^9–O^7 transesterification to occur. Our proposed biosynthetic pathway is premised on the ability of retronecine O^9 ester PAs, with small esterifying acids such as found in the insect-PAs, to transfer the esterifying acid to the O^7 position to allow the C^9 hydroxyl to be oxidized to an aldehyde. In this context, it is interesting to note that we found that the desoxy insect-PAs, **11** and **12,** do not give rise to danaidal in *C. transiens*, possibly because there is no C^7 hydroxyl group to facilitate exposure of the C^9 hydroxyl to oxidation. Esterase hydrolysis of the O^9 ester to gain access to the C^9 hydroxyl is apparently not an option, and this is not unexpected because plant-PAs, and to a lesser extent insect-PAs, have sterically hindered ester linkages that increase both their resistance to detoxication by esterases and their toxicity (Edgar et al., unpublished data).

Our biosynthetic pathway (Scheme 1, solid arrows) finds support in studies on the mechanism of epimerization of heliotrine (**5**) to 7*R-epi*-heliotrine (**6**) in *C. transiens* (Scheme 1; Schulz et al.1993). The epimerization was found to proceed via 7-ketoheliotrine (**13**), a structural analogue to **20**. Schulz et al.1993 also reported finding **21** and **22** in all *C. transiens* feeding experiments. These structures are analogues of **23** and **9**, respectively, in our pathway. Thus, we surmise that without O^9–O^7 intramolecular transesterification, *C. transiens* would only be able to produce **22** and not **9**. By generating insect-PAs, able to undergo intramolecular transesterification from O^9 to O^7, *C. transiens* is able to expose the primary alcohol to oxidation and to produce **9**.

Hartmann et al. (2005a) reported formation of O^7, O^9 diester insect-PAs, the platyphorines, in the arctiine *E. acrea*, but only in females. The same diesters have been found previously in leaf beetles of the genus *Platyphora* feeding as specialists on plants that contain PAs (Hartmann et al. 2001; Pasteels et al. 2001). Neither female *E. acrea* nor leaf beetles make **9**, and we envisage that platyphorines arise because, in female moths and in leaf beetles, the free C^9 primary hydroxyl in the intermediate O^7 ester insect iso-alkaloids, e.g., equivalent to **8**, is not oxidized to the aldehyde **20** as in male *E. acrea* but is available to be re-esterified by a second insect-derived acid. The C^9–C^7 diester platyphorines are expected to be more toxic than monoester insect-PAs, so there is an evolutionary advantage in producing them.

The reactions proposed in our pathway are occurring in the PA *N*-oxides and not in the free bases depicted in Scheme 1. PAs are rigorously maintained by arctiines as *N*-oxides (Hartmann 1999; Naumann et al. 2002) because the *N*-oxides are not amenable to cytochrome P450 oxidation that is normally associated with aromatization of PAs *in vivo* to chemically reactive, biologically hazardous dihydropyrrolizine ("pyrrole") esters such as **14**. This is why the previously proposed conversion of **6** to **14** (Scheme 1) is unlikely. The point at which the *N*-oxide oxygen is lost in our proposed pathway and whether cytochrome P450 isozymes are involved in aromatization are issues for future determination. However, because the *N*-oxides of **20** and **24** are already at the same level of oxidation as the dihydropyrrolizines **23** and **9**, an alternative and more likely mechanism is "dehydration" of the *N*-oxides of **20** and/or **24** directly to **23** and **9**, respectively, as shown in Fig. 3. The electron withdrawing influence of the aldehyde group would facilitate this, and the conversion of the *N*-oxides of **20** to **23** and/or **24** to **9** (Fig. 3) may occur spontaneously. A bio-mimetic demonstration of the conversion of **24** *N*-oxide to **9** by dehydration is the conversion of retronecine (**2**) *N*-oxide to **9** in high yield by manganese dioxide oxidation that proceeds via **24** *N*-oxide (Culvenor et al. 1970b). Loss of the *N*-oxide oxygen, under the conditions of the MnO_2 reaction, can only be explained by spontaneous dehydration of the intermediate **24** *N*-oxide to give **9**. *In vitro*, the aromatization of PA *N*-oxides is facilitated by acetylation of the *N*-oxide group and subsequent facile elimination of acetic acid (Bull et al. 1968; Mattocks 1986). This application of the Polonovsky reaction has traditionally been used to convert PA *N*-oxides to dihydropyrrolizines (Culvenor et al. 1970a) and is used to detect PA *N*-oxides on TLC plates and paper chromatograms (Molyneux and Roitman 1980; Mattocks 1986).

A key aspect of our proposal is that the aldehyde **20** is generated before aromatization, thus avoiding the intermediacy of hazardous "pyrrole" esters such as **14** proposed by Schulz et al. (1993). The presence of an electron withdrawing carbonyl on the aromatic "pyrrole" ring greatly reduces the alkylating potential of the O^7 ester of **23**. Compound **23** is expected, from knowledge of analogous structures, e.g., 7-angeloyldanaidal (Culvenor et al. 1970b), to be considerably more stable and relatively biologically inert in contrast to intermediates such as **14**. 7-Angeloyldanaidal is, however, readily hydrolyzed by warm water to **9** (Culvenor et al. 1970b), mimicking the proposed conversion of **23** to **9**.

An important aspect that must be addressed in considering the pathway we are proposing for the conversion of **5** to **9** (Scheme 1, solid arrows) is the requirement for retention of stereochemistry at C^7 during hydrolysis of the C^7 ester linkages of either **20** and/or **23**. Retention of stereochemistry is expected in the hydrolysis of **20** to **24.** There is, however, a risk of racemization in the hydrolysis of **23**, where the electron withdrawing aldehyde group may participate. Indeed the *in vitro* hydrolysis of 7*S*-angeloyldanaidal by warm water

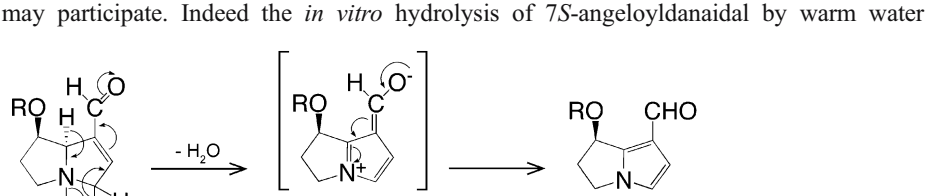


Fig. 3 Proposed mechanism for conversion of *N*-oxides of aldehydes **20** and **24** into dihydropyrrolizines **23** and **9**, respectively

resulted in racemization of the C^7 position (Culvenor et al. 1970b). Whether similar racemization occurs *in vivo* to the 7*R* analogue **23** needs to be determined by feeding synthetic **23** to *C. transiens* larvae.

It is clear from the results described above and the previous work of Hartmann et al. (2003b, 2005a) that one important role of insect-PAs, especially the creatonotines, e.g., **3**, is as mandatory intermediates in the production of hydroxydanaidal (**9**) by males of some arctiine species.

Broader Implications for PA-adapted Species Many Lepidoptera use hydroxydanaidal (**9**) as a male pheromone. In addition to the species of Danainae, Ithomiinae, and Arctiinae listed by Schulz (1998) and Schulz et al. (2004), **9** is also used by arctiines of *Haploa* (Davidson et al. 1997), *Pericallia*, *Amsacta*, and *Argina* (Boppré, unpublished) and probably others. The biosynthetic pathway to **9** proposed in this study for *C. transiens* may be employed by some of these. However, many species have not yet been investigated for insect-PAs let alone been the subject of biosynthetic studies on conversion of PAs to **9**. Very different biologies are involved, which are clearly the result of quite different origins, evolutionary pathways, and selection pressures, so that there are likely to be several biosynthetic mechanisms for producing **9** in PA-adapted species. Trigo et al. (1996) have, however, reported insect-PA-like substances in some ithomiine butterflies, raising the possibility that some of these species may also produce hydroxydanaidal (**9**) via insect-PA intermediates as shown in Scheme 1.

Another biosynthetic pathway for producing **9**, which avoids hazardous "pyrrolic" ester intermediates such as **14**, can be deduced from the use of viridifloric acid derived lactones **25** and **26** as pheromones by certain ithomiine and danaine butterflies (Edgar et al. 1976; Nishida et al. 1996; Schulz et al. 2004). These species may gain access to the C^9 hydroxyl by intramolecular transesterification reactions from lycopsamine (**19**), either directly in the case of lactone **25** or via an intermediate **27** in the case of lactone **26**. The retronecine (**2**) *N*-oxide released in each case could then be oxidized to **24**-*N*-oxide, and as previously proposed, this would yield **9** by spontaneous dehydration (Fig. 3).

Coremata Morphogenesis As a by-product of our experiments, we have shown that supinine (**10**) and/or insect-PAs **11** and **12**, although not precursors of the male pheromone **9**, may act as morphogens for development of the coremata. This shows that the size of the coremata does not indicate the amount of **9** a male possesses. In this new light, coupling vs non-coupling of coremata morphogenesis and pheromone biosynthesis and the specificity of inducing corematal growth require detailed quantitative studies (Boppré et al., unpublished data).

Acknowledgments Thanks to Monika Siegel and Anita Kiesel for technical support and Steven M. Colegate for LC-ESI-MS confirming molecular weight data.

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extracted and identified compounds from the cuticle of workers, the postpharyngeal gland of workers, ant pupae and larvae, and the fungal symbiont of three species of higher attine ants: *Atta colombica*, *Acromyrmex echinatior*, and *Sericomyrmex amabilis*. The relative proportions of identified compounds were compared and represented 11 classes: *n*-alkanes, alkenes, branched methylalkanes, branched dimethylalkanes, trimethylalkanes, branched alkenes, aldehydes, alcohols, acetates, acids, and esters. The chemical profiles in all three species are likely to be sufficiently different to allow discrimination at the species and colony level and sufficiently similar within colonies to generate a relatively constant colony-specific chemical gestalt. The relative likelihood of individual compounds being derived from the ants, the ant brood, or the fungal symbiont are discussed. We hypothesize that hydrocarbons are particularly important as recognition cues because they appear to simultaneously allow the assessment of developmental stages and the identification of symbiont, colony, and species.

Keywords Cuticular hydrocarbons · Colony odor · Recognition · Symbiosis

Introduction

Cuticular hydrocarbons profiles are thought to provide the main nestmate recognition templates in social insects (Singer 1998; Lahav et al. 1999), and many studies have shown that these compounds are involved in recognition processes between individuals, castes, and colonies (Vander Meer and Morel 1998). A colony's "odor profile" is typically determined by specific proportions of a series of hydrocarbons that function as a blurred barcode and prompts colony members to expel non-nestmate individuals with a different odor profile (Wilson 1971; Breed 1983). The origin of the chemical compounds involved in recognition and discrimination processes may differ across species, both for idiosyncratic reasons and because of variation in the genetic (Hölldobler and Wilson 1990; Beye et al. 1997, 1998; Boomsma et al. 2003) and environmental components (Obin 1986; Crosland 1989) of odor profiles. Environmental factors that contribute to colony odor include ambient temperature, humidity, soil characteristics, food, and nest material (Jutsum et al. 1979; Obin 1986; Crosland 1989; Heinze et al. 1996; Singer and Espelie 1996; Nielsen et al. 1999; Liang and Silverman 2000; Silverman and Liang 2001; Richard et al. 2004; Howard and Blomquist 2005).

The fungus-growing ants are exclusively a New World tribe of myrmicine ants (Attini) and are restricted largely to the tropics and subtropics of Central and South America. The attine tribe contains 13 genera and more than 210 species (Brandão and Mayhé-Nunes 2001; Mueller et al. 2001). The tribe is subdivided into a derived monophyletic group called the "higher attines," which includes the genera *Atta*, *Acromyrmex* (including *Pseudoatta*), *Trachymyrmex*, and *Sericomyrmex*, and a largely paraphyletic group called the "lower attines" that contain the remaining genera (*Apterostigma*, *Cyphomyrmex*, *Mycetarotes*, *Mycetophylax*, *Mycetosoritis*, *Mycetoagroicus*, *Mycocepurus*, and *Myrmicocrypta*; Chapela et al. 1994; Schultz and Meier 1995). While leaf-cutting ants collect fresh leaves for fungiculture, lower attines use feces or dead plant or arthropod material as substrate for their fungus gardens. Colony size is relatively small (around 10–100 workers) in the lower attines compare to the thousands of workers in a *Sericomyrmex* colony, the tens of thousands in an *Acromyrmex* colony, and the millions of workers in an *Atta* colony (Weber 1972; Hölldobler and Wilson 1990). All attine ants cultivate fungi that belong to the tribe

Leucocoprineae; the association is believed to have originated in the Amazon Basin more than 50 million years ago (Chapela et al. 1994; Mueller et al. 1998). The symbiosis is obligate and has at least diffusely coevolved with respect to several nutritional, physiological, and antibiotic characteristics (Mueller 2002; Currie et al. 2003, 2006; Richard et al. 2005; Mikheyev et al. 2007).

Previous work on *Acromyrmex* species has shown that the workers and fungus gardens of the same colony have partly similar and partly different chemical profiles (Viana et al. 2001; Richard et al. 2004, 2007) and that nestmate recognition within and across closely related species is related to differences in surface chemistry (Lambardi et al. 2007; Richard et al. 2007). The purpose of the present study was to further investigate the magnitude and variability of these differences by: (1) comparing the qualitative and quantitative differences among three different sympatric species that belong to different genera of higher attine ants (*Atta*, *Acromyrmex*, and *Sericomyrmex*) and (2) comparing the chemical profiles of different colony members and developmental stages (workers, worker PPGs, brood, and the fungus garden) within and between these ant genera.

Methods and Materials

Biological Material We used five colonies of *Atta colombica* (one without larvae), nine colonies of *Acromyrmex echinatior* (one without larvae; colony number Ae48, Ae109, Ae124, Ae132, Ae168, Ae210, Ae219, Ae221, Ae223), and four colonies of *Sericomyrmex amabilis* (two without larvae and pupae; colony numbers Sal220402-1, Sal210402, Sal110601-18, Sal030603-1). All colonies were collected in Gamboa, Panama, between 1994 and 2002, and had been maintained in the laboratory for a minimum of 2 years on a similar diet. At the time of sampling (July 2004), nest boxes were maintained at the University of Copenhagen under standardized conditions of approximately 70% relative humidity and 25°C. Colonies were provided with a diet of bramble leaves and rice grains, except for *Sericomyrmex* colonies that were provided with dried flower petals and small pieces of cut bramble leaves.

Chemical Analyses of Colonies Extracts were obtained from 200 mg of larvae or pupae or ten workers immersed in 1 ml of pentane for 10 min. Another ten workers from each colony were frozen for later dissection of their postpharyngeal glands (PPGs). Fragments of fungus garden were taken from all colonies of *Sericomyrmex* with two replicates per colony and from all colonies of *Atta* and *Acromyrmex* with four replicates per colony. To minimize any effects of leaf material on the chemical profiles, the fungus garden samples were taken from the mature middle section of fungus gardens where all leaf material was degraded (cf. Richard et al. 2007). All brood and workers were removed from the fungus fragments before chemical extraction, which was performed on 200 mg of fungus material immersed in 3 ml pentane for 10 min.

Extracts were stored at −20°C until analyzed. Before analysis, any remaining pentane was allowed to evaporate, and dried extracts were redissolved in 50 μl pentane containing 10 ng/μl eicosane (n-C_{20}) as an internal size standard. Two microliters of these mixtures were injected into a gas chromatograph coupled with a mass spectrometer, and compound quantification and identification were performed on an HP6890 gas chromatograph with an HP5973 mass-selective detector, equipped with an HP-5 column (30 m×0.32 mm inner diameter, film thickness=0.25 μm). The initial temperature was 80°C for 1 min with a

subsequent gradual increase of 10°C/min until 200°C was reached and finally a gradual temperature increase of 3°C/min until the maximum temperature of 340°C was reached and maintained for 10 min. The mass spectrometer was operated at 70 eV, and scanning was done from 40 to 550 amu at 1.5 scans/sec.

Identification of Compound Classes The objective of this study was to compare the chemical profiles of different species of ants, their larvae, pupae, and the fungus—not to provide absolute identifications of all of the different compounds that were found. Cuticular hydrocarbons (alkanes and mono-, di-, and trimethyl alkanes) were characterized to class level by using diagnostic ions, comparisons with standard mass spectrometry (MS) databases, and by determination of Kovats indices (see Carlson et al. 1998; Lommelen et al. 2006). The position of the double bond in the alkenes was not determined because our purpose was not to identify specific compounds. The identification of other compounds was based on comparison of retention times and diagnostic ions with NIST/EPA/NIH 172 mass spectral libraries. Our chemical profiles were also compared with previous studies where we did identify the chemicals associated with leaf-cutting ant fungus gardens (Richard et al. 2007).

Statistical Analyses The chemical profiles of the different colonies were obtained by gas chromatography (GC)–MS analysis and were compared within and among species. Quantification and chemical profile characterization were done by GC-MS peak integration using the relative abundance of the various peaks. To test for differences in chemical profiles among different groups, we used factorial analyses and nested two-way analyses of variance (ANOVAs) having ant species, ant colony nested within species, and development stage as main factors (using arcsine square root [X] transformed individual peak areas of all identified compounds; Statistica 6.0, StatSoft®). To evaluate the relative contribution of ant species origin, ant colony, and developmental stage or symbiont origin to the profiles, we performed type II ANOVAs on each of these factors individually, both for all compounds present in the profiles and for the class of hydrocarbons alone. Data are presented as median±semi-interquartile ranges of the relative areas. To compare the relative quantities of the different compound categories between the different species, we used nonparametric Kruskal–Wallis tests on ranks.

Results

A total of 179 compounds, of which 139 hydrocarbons (16 *n*-alkanes, 22 alkenes, 49 Me-alkanes, 44 Dime-alkanes, and 8 Trime-alkanes), were identified (see Supplementary material: Appendix Table 1) and present in sufficient quantities to be analyzed for their contribution to the overall chemical profiles of the three focal species *A. colombica*, *A. echinatior*, and *S. amabilis* (Appendix Tables 2, 3 and 4). The cuticular profiles of worker ants contained a mixture of alkanes, alkenes, and mono- and dimethylalkanes with chain lengths ranging from C16 to C39 in *A. colombica*, C18 to C37 in *A. echinatior*, and C27 to C37 in *S. amabilis* (Appendix Tables 1, 2, 3, and 4). Considerable amounts of 5, 9+5, 11 Dime C35, and trimethylalkanes were present in *A. colombica* and four other compounds were specific for workers of *A. echinatior*: C20:2; C23:2, 11MeC21, and xMeC21. *S. amabilis* was characterized by the presence of 3MeC29 in all samples and 7MeC33 and 12,16+14,18 Dime C32 in all samples except larvae.

When evaluating compounds in the five major classes of hydrocarbons only, there were significant differences among the three species, with *A. colombica* having more alkenes and trimethylalkanes, *A. echinatior* having more alkanes, and *S. amabilis* having more methylalkanes and dimethylalkanes (Tables 1 and 3). No chemical compounds other than hydrocarbons were identified on *S. amabilis* worker cuticles, but *A. echinatior* cuticles contained an appreciable fraction of aldehydes, whereas *A. colombica* workers expressed small quantities of aldehydes, alcohols, and formates (Tables 2 and 3). Larval and pupal profiles consisted almost exclusively of hydrocarbons in all three species (Table 2). In general, brood profiles were less variable in chain length than worker profiles in *A. colombica* (C18 to C37) and *A. echinatior* (C18 to C33) and shorter than worker profiles in *S. amabilis* (C21 to C33). Finally, there was a striking difference between the very small total amounts of hydrocarbons on the bodies of larvae and pupae, relative to the much larger amounts on worker cuticles (Table 3). Thus, developmental stages and symbionts had a highly significant impact on all compound groups, while only some compound groups appeared affected by colony-of-origin (alkanes and alkenes) and ant species (hydrocarbons and branched alkanes). Because of the difficulty of standardizing tissue samples among groups, such absolute quantity estimates need to be interpreted with caution. However, the fact that the amount of compounds on larvae and pupae was an order of magnitude less than on worker cuticles and fungus garden material makes it reasonable to infer that these quantitative differences are real.

The fungus chemical profiles contained alkanes, alkenes, and methyl alkanes with chain lengths ranging from C18 to C36 for *A. colombica*, C18 to C33 for *A. echinatior,* and C25 to C35 for *Sericomyrmex* (Appendix Tables 1, 2, 3 and 4). The fungus garden methylalkanes were MeC23 and MeC26 for *A. colombica* and *A. echinatior*, and MeC27 to MeC35 for *S. amabilis*. Dimethylalkanes with chains ranging from DimeC33 to DimeC35 were found in *A. colombica* and DimeC31 to DimeC33 in *S. amabilis*, whereas trimethyl alkanes were only found in *A. colombica* fungus gardens. The diversity of chemical compounds in the fungus garden was higher than the diversity in brood, workers, and PPGs (see Supplemantary material: Appendix Tables 1, 2, 3, 4). However, hydrocarbons were more abundant than all other chemical classes in the fungi cultivated by *A. colombica* and *A. echinatior*, whereas the *S. amabilis* symbionts also had relative large amounts of acetates (Table 2).

Table 1 Relative proportions (median±semi-interquartile ranges) of alkanes, alkenes, methylalkanes, dimethylalkanes, and trimethyl alkanes based on the total cuticular hydrocarbon quantities in three species of higher attine ants

Species	Number of Colonies	Alkanes	Alkenes	Methylalkanes	Dimethylalkanes	Trimethylalkanes	Hydrocarbons (%)
Atta colombica	5	21.41± 2.99	29.13± 0.56	0.29±0.02	8.10±0.35	41.11±1.59	100
Acromyrmex echinatior	9	77.78± 5.48	19.72± 4.97	2.5±1.96	0		100
Sericomyrmex amabilis	4	31.98± 6.59	1.35± 0.68	38.75±4.31	26.39±2.67		100
H (*P*) (Kruskal–Wallis)		13.9 (<0.001)	12.6 (0.01)	9.5 (0.008)	16.4 (<0.001)		

Table 2 Relative proportions (median±semi-interquartile ranges) of 8 different classes of compounds for three species of higher attine ants

	Sample size replicate (number of colonies)	Hydrocarbons	Aldehydes	Acetates	Alcohols	Esters	Formates	ANOVA
Fungus								
Atta colombica	18 (5)	78.54±3.19	0.63±0.21	12.11±1.80	0.97±0.45	6.01±2.24	0.03±0.07	$F(5, I=96)=85.7$, $P<0.001$
Acromyrmex echinatior	34 (9)	62.09±8.03	2.04±0.41	8.62±3.09		5.22±1.92		$F(3, I=136)=996$; $P<0.01$
Sericomyrmex amabilis	8 (4)	41.15±8.88	2.72±0.23	32.53±6.84	7.09±2.33	8.89±1.46		$F(4, I=40)=31.4$; $P<0.001$
H (*P*) (Kruskal–Wallis)		29.2 (0.000)	33.8 (0.000)	17.5 (0.001)		9.6 (0.008)		
Cuticle								
Atta colombica	5 (5)	96.23±0.52	1.44±0.30		1.05±0.19		0.12±0.02	
Acromyrmex echinatior	9 (9)	89.73±4.43	8.01±3.22					
Sericomyrmex amabilis	4 (4)	100.00±0.00	0.00±0.00					
H (P) (Kruskal-Wallis)		12.7 (0.001)	14.5 (0.000)					
Brood								
Atta colombica	8 (4)	100						
Acromyrmex echinatior	15 (6)	100						
Sericomyrmex amabilis	4 (2)	100±0.68	0±0.25		0±0.43			
H (*P*) (Kruskal–Wallis)		1.92 (0.382)						

Factorial analyses showed that the chemical profiles of larvae and pupae lie between the profiles obtained from worker cuticles and the fungal symbionts (Fig. 1) and that PPG profiles are most different from those of the fungal profiles. Moreover, each of these developmental stage or symbiont components of the colony had its own specific chemical profile nested within the overall characteristic profile for the colony and species (Fig. 2). Type II ANOVAs were performed to evaluate how much of the variation in the complete chemical profiles and in the profiles including only hydrocarbons could be attributed to ant species, ant colony, and developmental stage or symbiont origin. While the species component only explained 0.34% (R^2) of the variation in the complete chemical profiles, colony-of-origin explained 21%, and developmental stage or symbiont origin explained 35% of the variance in profiles. When only hydrocarbon compounds were included in the analysis, 11.4, 23.7, and 23.0% of the variation could be attributed to species, colony, and developmental stage/symbiont origin, respectively. The low amount of variation explained by ant species in the former analyses indicates that the presence of abundant nonhydrocarbon

Table 3 Real compound quantities (median±semi-interquartile ranges; in ng) and the results of two-way nested ANOVAs, having ant species, colony origin nested within ant species, and developmental stage or symbiont origin as main factors

	All compounds	All hydrocarbons	Alkanes	Branched Alkanes	Alkenes	Other compounds
Fungus						
A. colombica	13,228.58± 5,909.74	17,124.14± 7,181.14	5,781.12± 3,111.77	2,471.77± 989.96	917.45±218.57	3,688.85± 1,241.83
A. echinatior	19,499.50± 10,145.7	12,039.65± 6,895.37	11,484.25± 6,816.53	49.82±29.20	453.15±202.69	2,740.1± 1,333.4
S. amabilis	19,135.51± 2,659.86	10,882.03± 3,043.71	7,554.82± 1,305.19	1,653.57± 1,154.80	18.92±18.14	9,789.6± 2,627.9
CUTICLE						
A. colombica	6,835.57± 2,152.40	11,098.51± 3,761.58	1,348.78± 503.28	3,233.24± 882.61	2,158.39± 582.95	180.13± 36.58
A. echinatior	753.13±259.31	612.98±221.94	436.99± 190.63	11.44±6.36	107.94±28.66	60.36±46.62
S. amabilis	2,091.57± 493.98	2,091.57± 493.98	790.67± 106.14	1,402.42± 315.26	30.86±21.55	–
PPG						
A. colombica	22,339.15± 10,202.6	38,079.19± 17,032.21	536.41± 299.80	15,870.55± 6,785.20	5,424.2± 2,941.7	72.01±68.15
A. echinatior	797.56±531.06	747.11±492.65	724.75±507.3	–	8.16±11.70	12.36±14.9
S. amabilis	7,852.12± 1,331.79	7,852.12± 1,331.79	45.88±37.19	7,712.5± 1271.61	106.08±44.37	–
PUPAE						
A. colombica	535.21±115.94	970.91±236.64	93.92±28.92	348.25±89.04	78.08±10.20	14.33±5.60
A. echinatior	44.55±28.17	44.55±28.17	7.13±6.73	–	38.60±15.96	–
S. amabilis	232.62±22.51	232.62±22.51	109.16±6.45	97.93±13.48	25.52±2.57	–
LARVAE						
A. colombica	366.40±82.23	648.43±135.50	103.66±30.56	191.11±26.80	58.88±20.76	12.74±4.10
A. echinatior	11.39±7.80	11.39±6.04	11.39±6.04	-	-	-
S. amabilis	161.40±16.19	156.07±13.53	111.43±13.26	37.59±0.65	7.03±0.38	5.33±2.66
ANOVA						
Species	$F_{2,\ 85}$=1.99 (0.142)	$F_{2,\ 85}$=10.92 (<0.001)	$F_{2,\ 85}$=0.79 (0.453)	$F_{2,\ 85}$=20.31 (<0.001)	$F_{2,\ 85}$=1.51 (0.227)	$F_{2,\ 85}$=0.4802 (0.621)
Colony (species)	$F_{16,\ 85}$=2.38 (0.006)	$F_{16,\ 85}$=1.45 (0.134)	$F_{16,\ 85}$=2.58 (0.003)	$F16_{,\ 85}$=1.55 (0.100)	$F_{16,\ 85}$=2.877 (0.001)	$F_{16,\ 85}$=1.898 (0.032)
Dev. stage	$F_{4,\ 85}$=13.20 (<0.001)	$F_{4,\ 85}$=7.94 (<0.001)	$F_{4,\ 85}$=8.71 (<0.001)	$F_{4,\ 85}$=16.47 (<0.001)	$F_{4,\ 85}$=20.89 (<0.001)	$F_{4,\ 85}$=46.25 (<0.001)
Dev. stage× species	$F_{8,\ 85}$=2.87 (0.007)	$F_{8,\ 85}$=4.14 (0.001)	$F_{8,\ 85}$=0.76 (0.634)	$F_{8,\ 85}$=7.95 (<0.001)	$F_{8,\ 85}$=3.822 (0.001)	$F_{8,\ 85}$=6.875 (<0.001)

F values given with degrees of freedom as subscripts and *P* values in parentheses

compounds, particularly in the fungal samples of all three species (Table 2), tend to swamp the species differences that emerge when hydrocarbons are analyzed alone.

Discussion

We have shown that: (1) a large variety of potential recognition compounds is present in the colonies of fungus-growing ants; (2) workers express mostly hydrocarbons, whereas fungus gardens have a number of additional compounds; (3) ants and fungal symbionts of the three species (genera) can be statistically distinguished based on their chemical profiles; and (4)

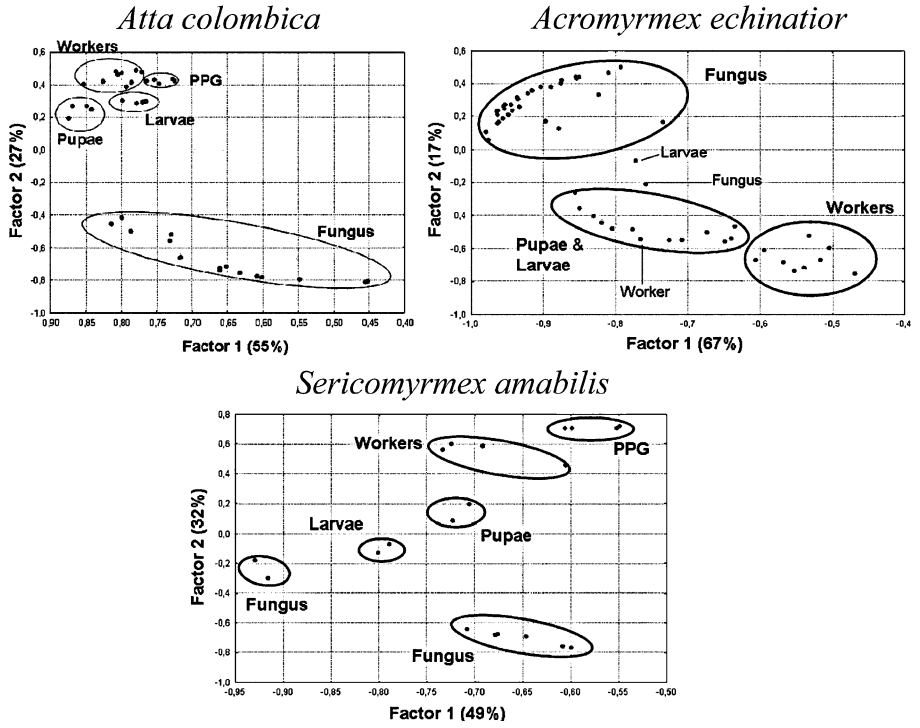


Fig. 1 Factorial analyses showing the differences in chemical profiles between worker cuticles, workers PPGs, pupal cuticles, larval cuticles, and fungus garden mycelium in *Atta colombica*, *Acromyrmex echinatior*, and *Sericomyrmex amabilis*. All analyses were based on the relative proportions of all chemical compounds identified by GC/MS. *Ellipses* have been drawn to emphasize the categories but have no specific statistical meaning

larvae and pupae have much smaller absolute quantities of cuticular hydrocarbons than workers. In the following sections, we discuss some of the implications of these results.

The Spectrum of Surface Chemicals in Fungus-growing Ants Both intra- and interspecific variation in the chemical profiles expressed by *A. colombica*, *A. echinatior*, and *S. amabilis* were found, but the intraspecific differences were much less than the interspecific differences both when complete and partial (hydrocarbons only) chemical profiles were considered. Moreover, species are characterized by some specific chemical compounds. Provisional data on *Atta cephalotes* (one colony: workers, larvae, and fungus) and *Acromyrmex octospinosus* (two colonies: workers, PPG, larvae, and fungus) from the same site in Gamboa, Panama, showed that differences between congeneric species are intermediate (Appendix Table 1). The species-specific chemical profiles are qualitatively similar among colonies (i.e., all colonies have the same compound composition), so that the differences obtained from multivariate statistics (Fig. 1) are mostly based on quantitative differences of the same compounds, except for the fungus gardens that harbor a number of unique nonhydrocarbon compounds. Differences in profiles were especially prominent among the hydrocarbons, which is consistent with these compounds having an important role in intra- and intercolony recognition (Lahav et al. 1999; Singer 1998; Wagner et al. 2000). Workers of all three species tended to have more (and more complex) hydrocarbons than the brood and the fungus gardens, which

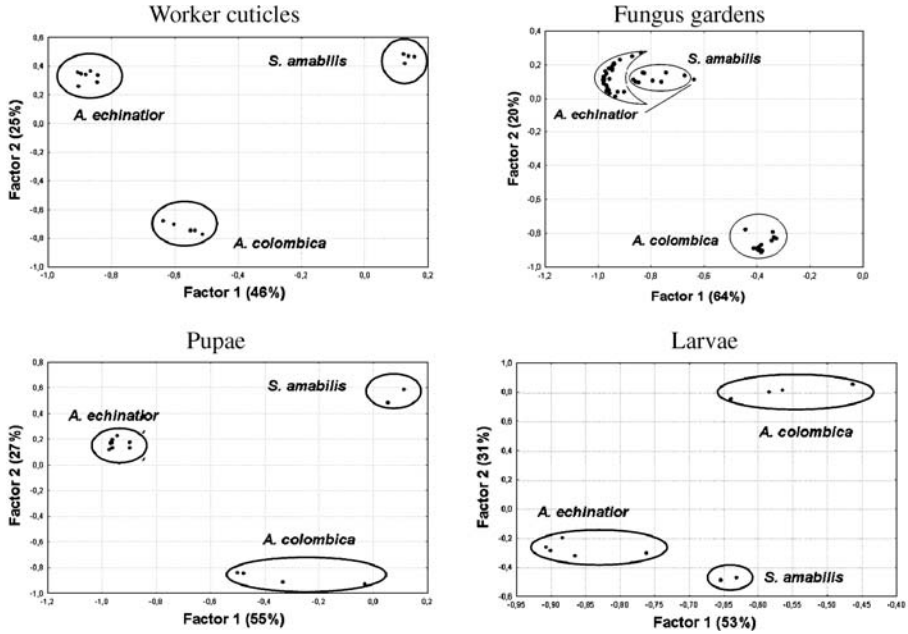


Fig. 2 Factorial analyses showing the differences in chemical profiles between the three species of fungus-growing ants *Atta colombica*, *Acromyrmex echinatior*, and *Sericomyrmex amabilis* for worker cuticles (*top left*), fungus gardens (*top right*), pupae (*bottom left*), and larvae (*bottom right*). All analyses were based on the relative proportions of all chemical compounds identified by GC/MS. *Ellipses* have been drawn to emphasize the categories but have no specific statistical meaning

generally have simpler profiles consisting mostly of *n*-alkanes and *n*-alkenes. Similar differences in worker and brood chemical composition have been observed in other ant species (*Myrmica* [Elmes et al. 2002], *Camponotus vagus* [Bonavita-Cougourdan et al. 1989], and *Lasius sakagamii* [Akino and Yamaoka 1998]). However, it is remarkable that the larvae and pupae of fungus-growing ants have so few cuticular compounds and such low quantities of them. This finding is unlikely to be an artifact as it matches an earlier result on leaf-cutting ants by Viana et al. (2001). Whether this adult-brood difference is typical only for fungus-growing ants or for ants in general remains to be determined.

Fungus Garden Chemistry The fungus produces several classes of compounds that ants do not synthesize, which confirms previous findings that workers only have alkanes, alkenes, and branched alkanes (Viana et al. 2001; Lambardi et al. 2007), whereas fungus gardens of *Acromyrmex* contain both aldehydes, alcohols, acetates, acids, and esters (Richard et al. 2007). The latter study that compared the fungus garden chemistry of Panamanian *A. octospinosus* and *A. echinatior* leaf-cutting ants showed that these fungi express the same chemical compounds but in different relative quantities among ant species and colonies (Richard et al. 2007). The same profiles, with the same chemical classes, were found in two colonies of *A. octospinosus* (Appendix Table 1), and the single colony of *A. cephalotes* likewise had a fungal profile that was similar to that of *A. colombica* both characterized by the presence of trimethylalkanes (Appendix Table 1). Richard et al. (2007) also showed that the fungus-derived compounds in *A. echinatior* and *A. octospinosus* have a, albeit weak, genetic component and that they are shared between the two species (see also Bot et al. 2001). As

Mikheyev et al. (2007) recently showed that the symbiont fungi of Panamanian *Atta* and *Acromyrmex* in Gamboa, Panama, are similar genetically, it appears that the *Atta* and *Acromyrmex* fungus gardens each express at least some compounds that are ant species specific but not shared by the ants. Given that all colonies were reared on the same laboratory diet, this suggests that the same fungi express different chemical (and thus possibly metabolic) traits depending on the ant genus that they are associated with. The degree to which symbionts of *Sericomyrmex* are related to those reared by *Atta* and *Acromyrmex* leaf-cutting ants is not clear (Chapela et al. 1994; Hinkle et al. 1994), but it seems unlikely that they belong to the same pool. Thus, the differences in chemistry between *Sericomyrmex* symbionts and symbionts of *Atta* and *Acromyrmex* leaf-cutting ants may, in part, be genetic.

Recognition in Fungus-growing Ants Differences in the proportion of hydrocarbons in workers (cuticle and PPG), brood, and fungus garden material are consistent with these compounds playing a central role in caste recognition, nestmate recognition, and discrimination between resident and alien strains of the fungal symbiont (Bot et al. 2001; Viana et al. 2001; Poulsen and Boomsma 2005; Richard et al. 2007). The patterns observed (Figs. 1 and 2) and the partitioning of variance in chemical profiles suggest that chemistry differences may allow efficient within-colony distinction between brood and symbionts without jeopardizing efficient nestmate recognition as hypothesized by Vander Meer and Morel (1998). This agrees with findings in other social insects; for example, cuticular hydrocarbons in the ant *Pachycondyla inversa* vary within and among colonies but also with an individual's social and reproductive status (Heinze et al. 2002). Similarly, cuticular hydrocarbons in the ant *Formica truncorum* vary among colonies, to some extent between patrilines within colonies, and among samples taken from the same colony in different seasons (Nielsen et al. 1999; Boomsma et al. 2003). In addition, in the termite *Reticulitermes virginicus* and the ant *C. vagus*, all castes carry the same hydrocarbons, but the caste-specific proportions differ (Howard et al. 1978, 1982; Blomquist et al. 1979; Bonavita-Cougourdan et al. 1989).

Specificity of the chemical profiles of workers, brood, and fungi is intriguing because these different components of the same insect society interact constantly. For example, workers could transmit colony odor to the fungal substrate when they chew the harvested leaves before integrating them into the garden. Other workers may regurgitate the content of their PPG (which is known to be a reservoir of colony gestalt odor profiles) to nestmates by a process called trophallaxis (Soroker et al. 1994, 1995). Grooming and other social interactions may further promote the circulation of hydrocarbons through the colony. Given these close interactions, it is interesting that the hydrocarbon compounds present in fungus material were always present on workers and brood but that the opposite is not true (Viana et al. 2001; Lambardi et al. 2007; Richard et al. 2007). Previous studies combined with our present data, therefore, indicate that hydrocarbons are transferred by workers when moving around in the garden while grooming fungus fragments and other workers, but whether the ants or the fungus are the ultimate producers of these compounds is not yet clear.

Acknowledgements We thank the Smithsonian Tropical Research Institute (STRI) for providing logistic help and facilities to work in Gamboa and the Autoridad Nacional del Ambiente y el Mar (ANAM) for permission to sample ant colonies in Panama and export them to Denmark. Fieldwork was supported by grants from the Carlsberg Foundation and the Danish Natural Science Research Council to JJB. All experiments performed in this manuscript comply with current Danish and USA law.

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Introduction

Moths are one of the most speciose insect groups with an estimated 185,000 species (Holloway et al. 1987). Cardé and Haynes (2004) speculated that one factor promoting this diversity is the ability to persist at low densities because of pair formation mediated by volatile female-produced sex pheromones. This hypothesis will be difficult to test; however, it is clear that the dominant paradigm among moths is that of long-distance attraction of males by female-produced sex pheromones. For example, Byers (2002) surveyed the published literature and estimated that attractant sex pheromones (or attractants known from empirical screening) have been identified in 1,572 moth species from 619 genera (not all major lineages are represented).

In their seminal discussion of chemical communication among animals, Wilson and Bossert (1963) suggested that volatile pheromones would have between 5–20 carbon atoms, minimal chemical complexity (e.g., few points of unsaturation, terminal acetate, alcohol, or aldehyde functional moieties), and a molecular weight between 80 and 300. Extrapolation from this model generated the predictions that: (1) in evolutionary time, the number of odorant molecules and thus species was unlimited, and (2) the number of sympatric moth species that could coexist would be defined by the number of unique pheromones used. Early models of reproductive isolation in moths were predicated on the latter prediction and emphasized the role of single compound, species-specific sex pheromones (e.g., Shorey 1970). As more moth sex pheromones were identified, it became clear that neither prediction was supported. The majority of moth pheromones are multi-component blends [the mean (±SE) number of components per species in 702 moth species was 1.86±0.05 (Byers 2002)], and sympatric species with similar phenologies and calling periodicities often share pheromone components (Arn 2001; Byers 2002, 2005, 2006).

The single species, single-pheromone component model of reproductive isolation was replaced by a model that predicts reproductive isolation as a result of the existence of species-specific blends of pheromone components (e.g., Löfstedt et al. 1991; Cardé and Haynes 2004). The principal prediction of this model is that individuals with pheromone blend ratios that deviate from the population mean will be less likely to attract mates (see Butlin and Ritchie 1989; Groot et al. 2006). The associated variation in mating success would impose stabilizing selection on the signal and constrain phenotypic evolution (Endler 1992). Yet, comparative studies of the sex pheromones of related species and populations show that moth sex pheromone signals vary (e.g., geographically) and are apparently less rigid than the stabilizing selection model predicts (e.g., Roelofs and Comeau 1969; Tumlinson et al. 1974; Cardé et al. 1977; Klun et al. 1979; Roelofs and Brown 1982; Löfstedt et al. 1991; Löfstedt and Kozlov 1996; McElfresh and Millar 2001).

Reconciliation of the observed patterns of moth sex pheromone variation and the species-specific pheromone component blends model requires a mechanism for directional selection capable of periodically overcoming the stabilizing effects of variation in mating success. Communication interference with sympatric moths that use similar blends of the same pheromone components is one putative mechanism. Groot et al. (2006) hybridized and backcrossed two moth species and measured the effects of communication interference between parental and backcross pheromone strains. Their data suggested that the resulting directional selection would be strong enough to overcome stasis in the pheromone blend. Evolutionary change in the pheromone blend as a result of communication interference requires the existence of heritable genetic variation in the pheromone blend.

The female-produced sex pheromone of the almond moth, *Cadra cautella*, is a two-component blend of (*Z,E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12–14:Ac, the major compo-

nent capable of inducing attraction alone) and (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac, the minor component, which is unattractive alone but augments attraction of the major component; Brady et al. 1971; Read and Haines 1979). Previous study of the pheromone biology of this species has documented high levels of phenotypic variation in the sex pheromone, narrow-sense heritabilities of 1.2±0.32, 0.75±0.24, and 0.46±0.17 for the titer of *Z*9,*E*12–14:Ac, *Z*9–14:Ac and pheromone blend ratio, and stabilizing male preference functions in wind-tunnel choice assays (Allison and Cardé 2006, 2007). These observations suggest that, whereas the pheromone blend ratio in *C. cautella* may experience stabilizing selection, it should respond to directional selection, a hypothesis tested in this study. The selected lines were maintained for five generations after the discontinuation of selection and sampled the last three to estimate the stability of the selected change in the ratio of the two sex pheromone components.

Methods and Materials

Insect Rearing and Pheromone Extraction and Analysis Selection lines were reared by using the same protocol and conditions as the general laboratory colony in a controlled environment rearing room. The laboratory colony used to initiate the selection lines and the rearing protocol and conditions has been previously described (Allison and Cardé, 2006). Temperature, humidity, and the light–dark regime were verified periodically with a Hobo® sensor (Onset Computer, Bourne, MA, USA). The ratio and titer of pheromone components was measured by gas chromatographic (GC) analysis of solvent extracts of pheromone glands excised from 1-d-old virgin female *C. cautella* during the second hour of the scotophase with procedures described in Allison and Cardé (2006). The mean phenotype (ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac) of each family was estimated from a pooled extract of the pheromone glands of five full-sibling females. Although this procedure greatly reduces GC analysis time, the contribution of each individual to the family mean is weighted by the amount of pheromone produced. The validity of this procedure for determining family pheromone characteristics was verified by Collins and Cardé (1989).

Selection Lines and Regime The aim of our selection protocol was to establish lines with high and low ratios of *Z*9,*E*12–14:Ac to *Z*9–14:Ac. Analysis of female pheromone gland content post-mating and oviposition is not practical because post-mating pheromone gland titers are low and unlikely to be representative of pre-mating titers and ratios (Raulston et al. 1975; Webster and Cardé 1984; Raina et al. 1986; Tang et al. 1992; but see Svensson et al. 2002). Excision and extraction of the female pheromone gland is a destructive process, and as female almond moths mate only once (J.D.A., personal observation), it was not possible to analyze the contents of the pheromone gland from a female and collect her progeny. Therefore, individual selection was not possible, and we used sib selection, a form of family selection in which the selected individuals have not contributed to the estimate of their family mean but are siblings of the individuals that were measured (Falconer and Mackay, 1996).

The parental generation of each selection line was initiated with 24 families formed of a single male–female pair (1–3 d old) chosen randomly from the base laboratory colony. The progeny of each pair was reared on 50 g of artificial diet (see Allison and Cardé, 2006). Last instars were collected, sexed (male testes are easily seen through the dorsal larval cuticle), and transferred to 180-ml plastic cups for pupation (larvae were kept together but separated by sex). The ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac produced by female

progeny of each family was measured by GC analysis of a pooled extract of five full-sib female progeny. High, low, and control lines were each started simultaneously by using this protocol and cumulatively were considered a replicate. There were three replicates started on consecutive weeks. The high, low, and control lines from all replicates were housed in the controlled rearing room described in Allison and Cardé (2006). Approximately 20 female and 10 male last instars were collected from each family each generation for the determination of the family phenotype and (if selected) propagation of the line. The collected larvae were kept on artificial diet as described above in a light box kept in the controlled environment rearing room. There was a separate light box for each selection regime, each of which was offset so that the second hour of the scotophase occurred first in the high regime light box and 3 and 6 h later in the low and control regime light boxes. The light boxes were identical, and Hobo® sensors (Onset Computer) were used periodically to verify rearing conditions. Determination of which families would contribute to the next generation had to be done while the remaining siblings were still reproductively viable (about 3–4 d). Once the lines were established, subsequent generations of the selected lines were propagated by crossing males and unsampled females from the six families with the highest (or lowest) ratios of *Z*9,*E*12–14:Ac to *Z*9–14:Ac. Subsequent generations of the control lines were propagated similarly except that the families were chosen randomly. To minimize inbreeding, siblings were never crossed, and six families were chosen independent of the number of families sampled each generation (Table 1).

Post-selection Selection in the high and low lines was discontinued after the F5. All of the selection lines (including the control lines) were reared following the same protocol described above without selection (i.e., pairs contributing to the next generation were randomly chosen without any knowledge of pheromone blend). The absolute and relative

Table 1 Number of families and daughters of *Cadra cautella* (mean±SE) sampled according to line and generation

Line	Generation					
	Parental	F1	F2	F3	F4	F5
High line 1	20 (5.00±0)	18 (4.44±0.22)	21 (5.10±0.15)	19 (5.00±0.08)	21 (4.76±0.10)	23 (4.87±0.13)
High line 2	20 (4.95±0.11)	20 (4.90±0.07)	18 (5.00±0.08)	20 (4.80±0.14)	20 (4.90±0.12)	20 (5.00±0)
High line 3	21 (5.05±0.11)	18 (4.50±0.26)	16 (4.89±0.09)	18 (4.83±0.12)	20 (4.80±0.14)	21 (4.90±0.10)
Control line 1	21 (4.90±0.10)	21 (4.71±0.18)	22 (5.05±0.10)	16 (4.94±0.14)	18 (4.78±0.13)	16 (4.81±0.19)
Control line 2	20 (4.95±0.14)	24 (4.75±0.26)	21 (4.86±0.10)	18 (4.44±0.18)	19 (5.00±0)	16 (4.94±0.06)
Control line 3	23 (4.74±0.18)	20 (4.85±0.08)	18 (5.06±0.06)	21 (4.95±0.05)	17 (5.00±0)	19 (4.79±0.12)
Low line 1	24 (5.04±0.04)	22 (4.68±0.20)	19 (5.21±0.21)	19 (4.68±0.15)	19 (4.89±0.11)	18 (4.78±0.13)
Low line 2	22 (4.77±0.11)	21 (4.90±0.07)	20 (5.10±0.10)	19 (4.89±0.11)	16 (5.00±0)	21 (4.76±0.14)
Low line 3	19 (4.95±0.05)	21 (4.91±0.15)	21 (4.76±0.12)	14 (5.00±0)	15 (5.00±0)	19 (4.89±0.07)

pheromone titers of each selection line were sampled as described above in generations F8, F9, and F10 and compared to titers in the last selected generation.

Statistics The effects of selection regime on the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac in female *C. cautella* were analyzed by analysis of covariance (ANCOVA) by using generation as the primary covariate. Initial analyses used the complete model (i.e., all three selection regimes: high, low, and control). A significant generation by selection regime interaction would indicate divergence among lines in the pheromone blend ratio. Subsequent ANCOVA analyses tested for a generation by selection regime interaction within each replicate by comparing each selected line to the control line from the same replicate with a Bonferroni corrected *P* value (0.05/6). Analysis of variance was used to test for significant differences in the component ratios and titers among generations within selection lines. Differences between specific generations were evaluated with the Students–Newman–Keuls' multiple range test (Steel and Torrie 1980). The narrow-sense heritability observed in each selection line (realized heritability) was estimated if a significant response to selection was observed. This estimate was based on the slope of the regression line of the cumulative selection differential on generation mean.

Results

Effect of Selection Regime on Pheromone Blend Ratio Initial ANCOVA analyses showed a highly significant generation by selection regime interaction for each replicate (replicate 1, $F_{2,352}=41.7$, $P<0.001$; replicate 2, $F_{2,349}=48.6$, $P<0.001$; replicate 3, $F_{2,336}=25.7$, $P<0.001$).

Selection for Higher Blends Artificial selection resulted in a significant increase in the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac in two of the three lines selected for high ratios (replicate 1, $F_{1,232}=31.0$, $P<0.001$; replicate 2, $F_{1,232}=30.9$, $P<0.001$; replicate 3, $F_{1,229}=2.5$, $P=0.11$; Fig. 1; Table 2). In high lines 1 and 2, the mean ratio (±SE) went from 13.72±1.02:1 to 20.13±0.68:1 and 9.87±0.54:1 to 15.89±0.85:1; a significant increase in the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac had occurred by the F2 in high line 1 and the F3 in high line 2. A significant response to selection did not occur in high line 3.

The observed increases in ratio reflect an increase in the titer of *Z*9,*E*12–14:Ac relative to *Z*9–14:Ac and could be as a result of an increase in the absolute titer of *Z*9,*E*12–14:Ac, a decrease in the absolute titer of *Z*9–14:Ac, or some combination thereof. It is clear that, in high lines 1 and 2, it was the titer of the major pheromone component (*Z*9,*E*12–14:Ac) that increased (Table 2; Fig. 2), whereas the minor component remained stable (Table 2; Fig. 3). The increase in the ratio of *Z*9,*E*12–14:Ac: *Z*9–14:Ac therefore was caused by an increase in the absolute titer of *Z*9,*E*12–14:Ac.

Randomly Selected Control Lines There was no significant change in the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac between the unselected parental generation and the last selected generation in any of the control lines (Table 3). There was a significant effect of generation on pheromone blend ratio in control line 3 (Table 3), although the parental and F5 generation did not differ. In control line 1, the titer of the major pheromone component *Z*9,*E*12–14:Ac increased 45% from 21.41±1.44 ng to 31.27±2.17 ng, whereas concurrently, the titer of the minor pheromone component also increased 45% from 2.23±0.15 to 3.21±

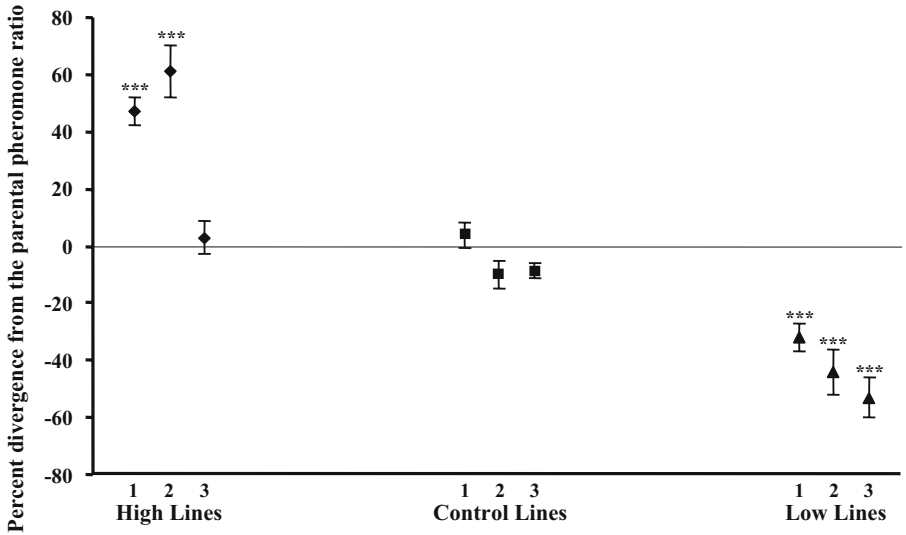


Fig. 1 The mean percent divergence (±SE) in the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac from the mean parental ratio in the last generation of each line of *Cadra cautella*. *Diamonds* represent the lines selected for high ratio, *squares* the unselected control lines, and *triangles* the lines selected for low ratio. Lines in which a significant effect of generation on the pheromone blend ratio was observed are designated by *asterisks* ($P<0.001$)

Table 2 Pheromone titers and ratios (mean±SE) of *Z*9,E12–14:Ac (I) to Z9–14:Ac (II) of the high selected lines of Cadra cautella by generation

Trait	Generation					
	Parental	F1	F2	F3	F4	F5
High line 1						
Ratio of I to II	13.72±1.02 A	12.98±1.04 A	16.65± 0.81 B	18.42±0.85 BC	19.79±0.85 C	20.13±0.68 C
Titer of I	21.58±1.30 A	19.15±1.76 A	33.62±1.89 B	35.96±2.29 B	5.06±2.29 B	33.71±1.82 B
Titer of II	1.72±0.15 A	1.6±0.18 A	2.04±0.45 A	1.97±0.10 A	1.85±0.10 A	1.71±0.10 A
High line 2						
Ratio of I to II	9.87±0.54 A	12.53±0.61 AB	11.24±0.55 A	14.64±0.74 BC	6.03±0.82 C	15.89±0.85 C
Titer of I	16.74±1.59 A	18.39±1.25 A	20.05±1.10A	25.29±1.4 B	29.06±1.51 B	26.83±1.67 B
Titer of II	1.71±0.21 A	1.71±0.16 A	1.82±0.10 A	1.66±0.12 A	1.91±0.15 A	1.81±0.16 A
High line 3						
Ratio of I to II	14.78±0.82 A	14.65±0.78 A	13.35±0.61 A	12.13±0.77 A	14.92±0.80 A	15.25±0.82 A
Titer of I	22.98±1.17 AB	25.84±2.27 AB	24.5±1.74 AB	26.09±1.78 AB	26.52±1.14 A	19.87±1.31 B
Titer of II	1.65±0.12 AB	1.9±0.21 AB	1.87±0.16 AB	2.09±0.11 A	1.9±0.15 AB	1.41±0.13 B

Means within rows not followed by the same letter are significantly different, Student–Newman–Keuls' multiple range test ($\alpha=0.05$).

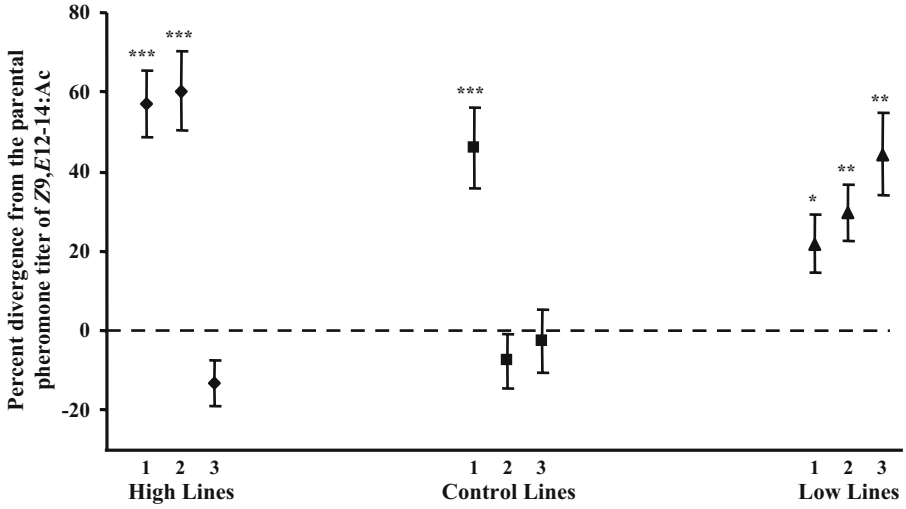


Fig. 2 The mean percent divergence (±SE) in the titer of *Z*9,*E*12–14:Ac from the mean parental titer for each line after five generations of selection in *Cadra cautella*. *Diamonds* represent the lines selected for high ratio, *squares* the unselected control lines, and *triangles* lines selected for low ratio. Lines in which a significant effect of generation was observed on the titer of *Z*9,*E*12–14:Ac are designated by *asterisks* (**P*< 0.05; ***P*<0.01; ****P*<0.001)

0.29 ng (Table 3). This parallel change in titers of both pheromone components with no concurrent change in component ratio is consistent with the previously reported significant additive genetic correlation between component titers (Allison and Cardé, 2006). In control lines 2 and 3, there was no change in the titers of either pheromone component.

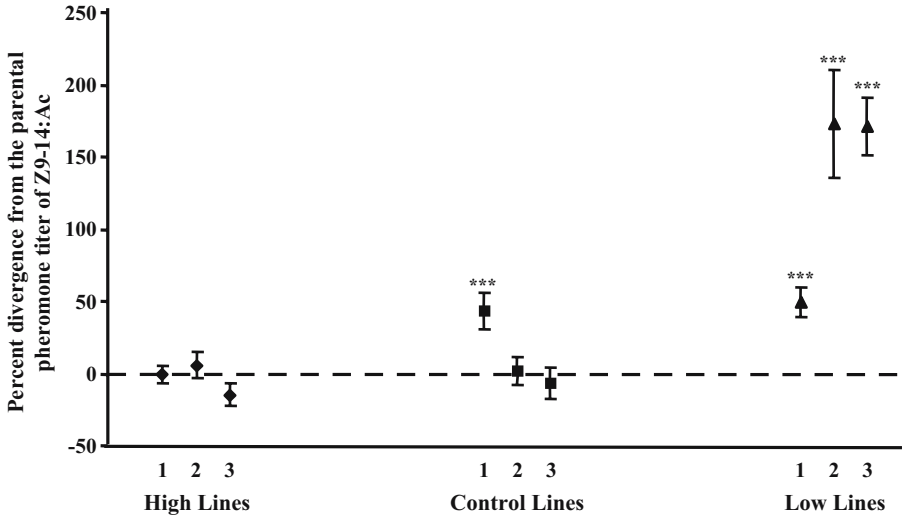


Fig. 3 The mean percent divergence (±SE) in the titer of *Z*9–14:Ac from the mean parental titer for each line after five generations of selection in *Cadra cautella*. *Diamonds* represent lines selected for high ratio, *squares* unselected control lines, and *triangles* lines selected for low ratio. Lines in which a significant effect of generation was observed on the titer of *Z*9–14:Ac are designated by *asterisks* (*P*<0.001)

Table 3 Pheromone titers and ratios (mean±SE) of *Z*9,*E*12–14:Ac (I) to *Z*9–14:Ac (II) of the control lines of *Cadra cautella* by generation

Trait	Generation					
	Parental	F1	F2	F3	F4	F5
Control line 1						
Ratio of I to II	9.88±0.50 A	9.83±0.45 A	10.44±0.58 A	10.39±0.50 A	10.50±0.57 A	10.23±0.53 A
Titer of I	21.41±1.44 A	27.87±1.57 B	30.02±1.78 B	29.58±1.62 B	28.61±1.77 B	31.27±2.17 B
Titer of II	2.23±0.15 A	2.95±0.20 AB	3.10±0.29 AB	2.93±0.17 AB	2.85±0.22 AB	3.21±0.29 B
Control line 2						
Ratio of I to II	12.72±0.70 A	10.28±0.50 A	12.10±0.38 A	11.79±0.74 A	11.93±0.57 A	11.47±0.97 A
Titer of I	25.73±1.95 A	23.68±1.28 A	28.90±1.99 A	26.38±2.07 A	29.42±2.27 A	23.75±1.71 A
Titer of II	2.17±0.23 A	2.33±0.15 A	2.47±0.18 A	2.27±0.21 A	2.58±0.22 A	2.21±0.20 A
Control line 3						
Ratio of I to II	11.80±0.59 AB	11.98±0.78 AB	10.77±0.47 A	13.04±0.65 BC	14.84±0.79 C	12.83±0.88 ABC
Titer of I	30.58±1.29 A	24.2±1.08 A	26.60±1.37 A	26.23±1.71 A	30.92±1.78 A	31.18±2.40 A
Titer of II	2.73±0.17 A	2.62±0.18 A	2.50±0.14 A	2.01±0.16 A	2.14±0.13 A	2.55±0.29 A

Means within rows not followed by the same letter are significantly different, Student–Newman–Keuls' multiple range test.

Selection for Lower Blends Artificial selection lowered the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac in all three of the low selected lines (replicate 1, $F_{1,232}=12.5$, $P<0.001$; replicate 2, $F_{1,233}=16.7$, $P<0.001$; replicate 3, $F_{1,223}=55.6$, $P<0.001$; Table 4). In low lines 1, 2, and 3, the mean ratio (±SE) changed from 10.74±0.78:1 to 7.35±0.41:1, 10.11±0.66:1 to 5.65±0.55:1 and 13.63±0.82:1 to 6.47±0.26:1 (Table 4; Fig. 1). It was not until the last, F4 and F1 generation that a significant decrease in ratio occurred in low lines 1, 2, and 3, respectively.

The titers of the individual pheromone components in all three of the low lines selected for decreased ratios of *Z*9,*E*12–14:Ac to *Z*9–14:Ac changed. The titer of the major pheromone component *Z*9,*E*12–14:Ac was significantly higher in the last selected generation than the unselected parental generation in low lines 1, 2, and 3 (Table 4; Fig. 2). At the same time, the titer of the minor pheromone component *Z*9–14:Ac also increased significantly in low lines 1, 2, and 3 (Table 4; Fig. 3). The decrease in the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac was caused by a greater relative increase in the titer of *Z*9–14:Ac than in the titer of *Z*9,*E*12–14:Ac.

Post-selection The mean pheromone blend ratio (±SE) decreased from the last selected generation (the F5) of high lines 1 and 2; five generations after selection was discontinued (the F10; there was no change in the pheromone blend ratio of high line 3; Table 5). Thus, in both high lines 1 and 2, the ratio of components had drifted (or been selected by unknown forces) down. In all three high selected lines, there was a significant decrease in the titer of *Z*9,*E*12–14:Ac within five generations of the termination of selection (Table 5). In high line 3, the titer of *Z*9–14:Ac was significantly lower in the F10, five generations after the termination of selection (Table 5). The titers of *Z*9–14:Ac in the F10 of high lines 1 and 2 did not differ from titers in the F5, the last selected generation.

Table 4 Pheromone titers and ratios (mean±SE) of *Z*9,*E*12–14:Ac (I) to *Z*9–14:Ac (II) of the low selected lines of *Cadra cautella* by generation

Trait	Generation					
	Parental	F1	F2	F3	F4	F5
Low line 1						
Ratio of I to II	10.74±0.78 A	9.93±0.70 AB	9.94±0.37 AB	8.89±0.62 AB	8.86±0.35 AB	7.35±0.41 B
Titer of I	17.52±0.95 A	22.30±0.87 B	32.83±1.42 D	27.88±1.73 C	23.85±1.35 B	21.47±1.26 B
Titer of II	1.99±0.21 A	2.48±0.17 AB	3.40±0.21 BC	3.71±0.61 C	2.90±0.2 ABC	3.01±0.20 ABC
Low line 2						
Ratio of I to II	10.11±0.66 A	8.84±0.46 A	8.97±0.52 A	8.39±0.72 AB	6.35±0.65 BC	5.65±0.55 C
Titer of I	17.87±1.04 A	22.67±1.46 B	26.00±1.59 B	23.10±1.24 B	23.60±1.13 B	22.98±1.26 B
Titer of II	1.95±0.18 A	2.71±0.21 A	3.09±0.26 AB	3.10±0.29 AB	4.23±0.38 BC	5.31±0.73 C
Low line 3						
Ratio of I to II	13.63±0.82 A	10.00±0.42 B	8.88±0.64 BC	6.97±0.30 C	7.74±0.48 BC	6.47±0.26 C
Titer of I	21.60±1.21 A	22.23±1.21 A	20.76±1.77 A	20.62±1.28 A	30.25±2.23 B	31.18±2.26 B
Titer of II	1.72±0.17 A	2.34±0.19 AB	2.38±0.26 AB	2.99±0.21 B	4.09±0.37 C	4.45±0.34 C

Means within rows not followed by the same letter are significantly different, Student–Newman–Keuls' multiple range test (α=0.05).

In control lines 1 and 3, significant differences between the mean pheromone blend ratio in the last selected generation and the F10 were not observed (although differences among some of the generations of control line 1 were observed; Table 6). The mean ratio in control line 2 decreased significantly from the last selected generation to the F10. A significant

Table 5 Pheromone titers and ratios (mean±SE) of *Z*9,*E*12–14:Ac (I) to *Z*9–14:Ac (II) of the high selected lines of *Cadra cautella* after relaxation of selection

Trait	Generation			
	F5, last selected generation	F8	F9	F10
High line 1				
Ratio of I to II	20.13±0.68 A	18.27±0.69 AB	17.25±1.26 AB	15.67±1.26 B
Titer of I	33.71±1.82 A	29.82±1.18 A	19.81±0.79 B	24.43±1.96 B
Titer of II	1.71±0.10 A	1.64±0.06 AB	1.22±0.09 B	1.59±0.10 AB
High line 2				
Ratio of I to II	15.89±0.85 A	11.81±0.68 B	10.83±0.39 B	8.06±0.45 C
Titer of I	26.83±1.67 A	19.39±0.90 B	18.52±0.73 B	17.93±1.34 B
Titer of II	1.81±0.16 A	1.75±0.13 A	1.72±0.07 A	2.28±0.21 A
High line 3				
Ratio of I to II	15.25±0.82 A	13.24±0.63 A	14.49±0.86 A	13.85±0.53 A
Titer of I	19.87±1.31 A	16.48±0.69 B	11.87±0.92 C	12.71±0.79 C
Titer of II	1.41±0.13 A	1.26±0.07 AB	0.87±0.10 B	0.92±0.06 B

Means within rows not followed by the same letter are significantly different, Student–Newman–Keuls' multiple range test (α=0.05).

Table 6 Pheromone titers and ratios (mean±SE) of *Z*9,*E*12–14:Ac (I) to *Z*9–14:Ac (II) of the control lines of *Cadra cautella* after relaxation of selection

Trait	Generation			
	F5, last selected generation	F8	F9	F10
Control line 1				
Ratio of I to II	10.23±0.53 AB	12.60±0.62 C	11.23±0.81 BC	8.53±0.47 A
Titer of I	31.27±2.17 A	22.64±0.82 B	17.28±1.44 B	16.41±1.29 B
Titer of II	3.21±0.29 A	1.87±0.12 B	1.56±0.12 B	1.93±0.11 B
Control line 2				
Ratio of I to II	11.47±0.97 A	9.14±0.34 AB	9.31±0.54 AB	7.78±0.63 B
Titer of I	23.75±1.71 A	20.52±1.35 AB	19.37±1.59 AB	15.82±1.04 B
Titer of II	2.21±0.20 A	2.25±0.13 A	2.16±0.25 A	2.16±0.21 A
Control line 3				
Ratio of I to II	12.83±0.88 A	9.56±0.27 A	10.39±0.83 A	9.82±0.57 A
Titer of I	31.18±2.40 A	26.56±0.90 A	21.37±1.29 A	22.51±1.96 A
Titer of II	2.55±0.29 A	2.81±0.15 A	2.12±0.17 A	2.32±0.19 A

Means within rows not followed by the same letter are significantly different, Student–Newman–Keuls' multiple range test (α=0.05).

decrease in the titers of *Z*9,*E*12–14:Ac (control lines 1 and 2) and *Z*9–14:Ac (control line 1) after five generations of relaxed selection were observed (Table 6).

The mean pheromone blend ratio in low line 2 decreased significantly after selection was stopped (Table 7) but not in low lines 1 and 3. The titer of *Z*9,*E*12–14:Ac decreased significantly after selection was discontinued in low line 2 (Table 7). The titer of *Z*9,*E*12–

Table 7 Pheromone titers and ratios (mean±SE) of *Z*9,*E*12–14:Ac (I) to *Z*9–14:Ac (II) of the low selected lines of *Cadra cautella* after the relaxation of selection

Trait	Generation			
	F5, last selected generation	F8	F9	F10
Low line 1				
Ratio of I to II	7.35±0.41 A	7.82±0.34 A	8.07±0.41 A	7.23±0.31 A
Titer of I	21.47±1.26 A	21.90±1.43 A	16.90±0.85 A	19.09±1.17 A
Titer of II	3.01±0.20 A	2.82±0.18 AB	2.17±0.15 B	2.64±0.12 AB
Low line 2				
Ratio of I to II	5.65±0.55 A	1.9±0.20 B	2.11±0.41 B	1.92±0.32 B
Titer of I	22.98±1.26 A	16.45±1.18 B	12.25±0.95 B	13.02±1.06 B
Titer of II	5.31±0.73 A	9.82±0.77 B	7.26±1.00 AB	7.48±0.71 AB
Low line 3				
Ratio of I to II	6.47±0.26 A	6.09 ± 0.17 A	7.05±0.24 A	6.33±0.34 A
Titer of I	31.18±2.26 A	24.77±0.90 AB	22.46±1.24 B	25.76±2.19 AB
Titer of II	4.45±0.34 A	4.12±0.17 AB	3.25 ± 0.26 B	4.20±0.46 AB

Means within rows not followed by the same letter are significantly different, Student–Newman–Keuls' multiple range test (α=0.05).

14:Ac did not differ between F10 or the last selected generation in either of low line 1 or 3. The titer of *Z*9–14:Ac did not differ between the F10 and the last selected generation in any of the low selected lines (although differences among generations were observed in all three lines).

Selection Differential The mean (±SE) selection differentials during the six generations of selection in high lines 1, 2, and 3 were 4.18±0.4, 3.40±0.32, and 3.59±0.39, respectively. Among the high selection lines, on average, the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac was 3.72±0.23 parts *Z*9,*E*12–14:Ac higher in the selected families than in the average high line family. The transient declines in ratio observed in the F1 of high line 1, F2 and F5 of high line 2, and F1, F2, and F3 in high line 3 cannot be attributed to low selection pressure. The mean (±SE) selection differentials for low lines 1, 2, and 3 during the six generations of selection were 2.64±0.39, 2.81±0.24, and 1.94±0.51, respectively. Among the low selection lines, the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac was on average 2.46±0.27 parts *Z*9, *E*12–14:Ac lower in the selected families than in the average low line family. The transient increases in ratio observed in the F2 of low line 1, F2 of low line 2, and F4 of low line 3 do not appear to be caused by low selection pressures. The mean selection differentials for the low and high lines differed significantly (t=3.54; P<0.05).

Realized Heritability The mean realized heritability of the lines that responded to selection was 0.74±0.03 and 0.80±0.26 for the high and low lines, respectively. The overall realized heritability for all five lines that responded to selection was 0.77±0.14. These estimates indicate that a large proportion of the phenotypic variance is caused by additive genetic factors and are higher than the narrow-sense heritability previously reported to the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac (0.46±0.17; Allison and Cardé, 2006).

Discussion

Few studies have examined the effect of artificial directional selection on moth sex pheromone production traits. Sreng et al. (1989) selected for high and low ratios of (*E*)-9-dodecenyl acetate to (*E*)-9-dodecenyl acetate plus (*E*)-11-tetradecenyl acetate in the redbanded leafroller, *Argyrotaenia velutinana* (Lepidoptera: Tortricidae) sex pheromone. In three generations of selection, the percent (*E*)-9-dodecenyl acetate went from approximately 24 to 14% and 28 to 42% in the low- and high-selected lines, respectively. As Sreng et al. (1989) point out, (*E*)-9-dodecenyl acetate is a minor pheromone component, and thus, the behavioral significance of this result is unclear. In the pink bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae), 12 generations of selection increased the mean (±SD) percentage of (*Z*,*E*)-7,11-hexadecadienyl acetate from 42.9±1.0 to 48.2± 1.2 (Collins and Cardé 1989), whereas six generations of selection increased the titer of both pheromone components from 24.0±8.1 to 45.8±7.9 ng per female (Collins et al. 1990). As Collins and Cardé (1989) point out, the observed change in blend ratio is likely transparent to male pink bollworm moths because of their broad response spectrum.

In this study, selection for a novel ratio of *Z*9,*E*12–14:Ac to *Z*9–14:Ac was successful in all three of the low lines and two of the three high selected lines. Change in the ratio of pheromone components occurred via a different mechanism between selection regimes and the same mechanism within selection regimes. In the two high lines that responded to artificial selection, selection for high ratios resulted in a significant increase in the titer of *Z*9,*E*12–14:Ac with no concomitant change in the titer of *Z*9–14:Ac. In all three of the low

selected lines, the titers of both pheromone components increased as a result of selection; however, the proportional increase in the titer of *Z*9–14:Ac was greater. The observed change in pheromone blend ratio is consistent with selection as a result of mate choice driving the evolution of sexual signals (Darwin 1871; Andersson 1994).

The preference function of male *C. cautella* is dependent on bioassay design. In no-choice trials, the probability that a male will initiate upwind flight and subsequently contact the pheromone point source is independent of the ratio of pheromone components tested. Independence of male orientation behavior and the pheromone blend ratio suggests the absence of discrimination in male *C. cautella*. Conversely, choice trials documented the predicted unimodal preference function, as male *C. cautella* preferred the median pheromone blend of 13 parts *Z*9,*E*12–14:Ac to 1 part *Z*9–14:Ac over lures baited with 0, 2, 6.6, and 20 parts *Z*9,*E*12–14:Ac to 1 part *Z*9–14:Ac and lures baited with just *Z*9,*E*12–14:Ac (Allison and Cardé 2007). In two of the low selected lines and one of the high selected lines, the mean female pheromone blend ratio moved beyond the point where males discriminate (i.e., 6.6 parts *Z*9,*E*12–14:Ac to 1 part *Z*9–14:Ac and 20 parts *Z*9,*E*12–14:Ac to 1 part *Z*9–14:Ac). These results demonstrate that not only can significant changes in mean pheromone blend occur in only a few generations, but that they could have significant fitness consequences on mate attraction.

Assuming the absence of genetic constraints, the response of a phenotypic trait to selection is defined by the equation ($R=h^2S$), where the response, R, is the product of the narrow-sense heritability of the trait (h^2) and the selection differential (S) (Roff, 1997). The realized heritability describes how the response to selection (R) is related to the selection differential (S) and ultimately how the trait responded to selection. Published estimates of realized heritabilities from selection experiments on moth sex pheromone production traits range between 0.50 and 1 (Collins and Cardé 1989; Sreng et al. 1989; Collins et al. 1990). Like the realized heritabilities observed in this study (0.74±0.03 and 0.8±0.26 for the high and low selected lines), these estimates suggest the existence of abundant heritable variation in the ratio of sex pheromone components in moths.

The shape of the mating preference of the responding sex (males in most moths) defines the relationship between a signal phenotype and the probability of the signal phenotype being accepted (see Ritchie 1996). Although few studies have examined mating preferences, both directional and stabilizing preference shapes have been observed (see Basolo 1996, 1998; Ritchie 1996; Ritchie et al. 2005; Allison and Cardé 2007). Both types of mating preference should deplete the amount of additive genetic variation available to selection (see Roff 1997). Male moths are expected to possess stabilizing mating preferences (see Butlin and Ritchie 1989; Groot et al. 2006), and choice assays in *C. cautella* document their existence (Allison and Cardé 2007). However, the results of this selection experiment demonstrate the existence of abundant additive genetic variance in the ratio of pheromone components.

It remains a major challenge to explain the maintenance of additive genetic variance in characters that experience strong stabilizing or directional selection (Barton and Turelli 1989; Roff 1997). Several mechanisms have been proposed (see Roff 1997), but to date it, has not been possible to determine which, if any, of these explain the abundant additive genetic variation in moth pheromone blends (see Allison and Cardé 2006). Alternatively, the assumption that moth sex pheromone blends experience strong stabilizing selection may be incorrect. Data in *C. cautella* are inconsistent with the existence of strong stabilizing selection [e.g., abundant additive genetic variance and male response data in no-choice trials that suggests male discrimination among naturally occurring pheromone blend ratios may not occur (see Allison and Cardé 2006, 2007)]. The possibility exists that the

importance of the pheromone blend in the maintenance of reproductive isolation in moths has been overstated, at least in *C. cautella*.

Pheromone evolution has relevance for both our understanding of the speciation process in moths (e.g., Linn and Roelofs 1995; White et al. 1995; Löfstedt and Kozlov 1996) and pest management (e.g., Cardé and Minks 1995; Cardé 2007). In part, interest in the identification of moth sex pheromones has been generated by the expectation that they could be used operationally in pest management programs. Direct (e.g., mass trapping, attract-and-kill formulations, and mating disruption) and indirect (e.g., monitoring of emergence and population levels) pheromone-based pest management tactics exist. Like chemical pesticides, the broad-scale implementation of direct pheromone-based pest management tactics will result in strong selection pressure. Because of the necessity of coordination between signaler and receiver, there is debate over the probability of the evolution of resistance to direct pheromone-based pest management tactics. Despite being used operationally for over 20 yrs against a variety of moth pests (see Cardé and Minks 1995; Cardé 2007), there has been only one published report suggestive of the evolution of resistance to a direct pheromone-based pest management tactic (Mochizuki et al. 2002). The results of the present study demonstrate that significant change in the pheromone blend ratio can occur in only a few generations of selection in *C. cautella*. Evaluation of selection favoring novel pheromone blend ratios as a potential mechanism for the evolution of resistance to direct pheromone-based pest management tactics will require further empirical study; however, these results suggest that it is a possibility.

Acknowledgments We acknowledge T. Berhane, K. Buhrmeister, and S. Roufian for reliable and invaluable technical assistance rearing insect colonies; Drs J. D. Hare, K. A. Justus, J. G. Millar, R. A. Redak, and D. A. Roff for helpful the comments and discussions, and NSERC Canada and the University of California, Riverside for financial support.

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Keywords *Ceratitis capitata* · Mediterranean fruit fly · Tephritidae · Mate choice · Ginger root oil · Pheromones

Introduction

Phytochemicals influence the mating behavior of phytophagous insects in two main ways. Specific compounds that occur in host plants may signal the presence of food or oviposition resources to females; males, responding to the same signals, high female traffic, or both, may aggregate at the same sites because they offer high probability of encountering potential mates (Thornhill and Alcock 1983; Bernays and Chapman 1994). Males of many phytophagous insects aggregate at the primary feeding and oviposition sites of females, and chemical stimuli may guide males, not only to the appropriate plant species (Quiroz et al. 2005) but also to plants or structures of a given host species that is preferred by females. For example, males and females of an oligophagous, desert-dwelling grasshopper tend to settle preferentially on those creosote bushes (the primary food resource) whose leaves contain relatively low amounts of the potent antioxidant nordihydroguaiaretic acid (Greenfield et al. 1987). Males of a longhorn beetle species respond to the odor of eucalyptus logs, where females aggregate for egg-laying (Hanks et al. 1996; see also Ginzel and Hanks 2005). Male attraction to host plant volatiles, leading to increased encounter rates with receptive, pre-ovipositing females, has also been reported in moths (Coracini et al. 2004) and bark beetles (Hovorka et al. 2005).

In addition to affecting male and female spatial distributions, plant chemicals may have a more subtle effect on insect mating systems through mediation of the production and release of sex pheromones (Landolt and Phillips 1997). For example, particular plant chemicals ingested by larvae (Connor et al. 1981; Lofstedt et al. 1989) or adults (Pliske 1975; Krasnoff and Dussourd 1989) may serve as precursors for pheromone synthesis. Feeding on the host plant may also stimulate production of male sex pheromone (Hughes and Renwick 1977), and exposure to host plant volatiles may trigger pheromone release (Jaffe et al. 1993). In addition, plant odors may affect female responsiveness to male sex pheromones. In various taxa, including flies (Bartelt et al. 1988), beetles (Dowd and Bartelt 1991), and moths (Landolt et al. 1994), female attraction to male pheromone is enhanced in the presence of host plant odors.

These main effects of plant chemicals—influencing mate location and intersexual communication—appear to be important in shaping the lek mating system (Prokopy and Hendrichs 1979) of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Tephritidae). Males aggregate in the canopy of host and nonhost trees and defend individual leaves as mating territories. Whereas perching on the leaf undersurfaces, males engage in 'calling behavior' in which the abdomen is curled upward, a bubble-like structure (the rectal epithelial sac) is everted, and a sex pheromone is emitted (Arita and Kaneshiro 1986). The pheromone is a complex blend that contains three to five major components known to be attractive to females (Heath et al. 1991; Jang et al. 1994) plus at least 50 minor components whose function has not yet been elucidated (Jang et al. 1989). After female arrival at a male perch, the male initiates vigorous wing movements coupled with side-to-side head rocking (Féron 1962). If the female remains stationary, the male mounts and attempts to copulate. Copulation generally lasts 2–3 hr (Whittier et al. 1992). Females are highly selective and reject courting males in more than 90% of laboratory observations by moving away during male signaling or dropping from the substrate after male mounting (Whittier et al. 1994).

Regarding intersexual encounter sites, field data (Hendrichs and Hendrichs 1990) showed that mating aggregations (leks) were non-randomly distributed among available host trees, with most matings (49%) occurring on orange trees (*Citrus sinensis* L.) although this host represented only a small fraction (12%) of all available host trees in the habitat. Furthermore, within the canopy of individual trees, both sexes settled in greater numbers in the vicinity of oranges than in locations having comparable physical characteristics (e.g., light level, temperature, and leaf density) but no fruits (Shelly and Kennelly 2007). Although visual stimuli are probably involved as well, strong attraction to citrus volatiles has been demonstrated for both sexes of *C. capitata* under field conditions (Katsoyannos et al. 1997).

Recent work has shown that plant-derived chemicals may also influence the mating success of male medflies. Papadopoulos et al. (2001) and Shelly et al. (2004a) found that *C. capitata* males exposed to oranges obtained significantly more copulations than non-exposed males. In one case, the exposed males obtained 66% of the total matings (Shelly et al. 2004a). Similarly, male medflies exposed to the bark and fruits of guava trees (*Psidium guajava* L.) had a mating advantage over males deprived access to these substrates (Shelly and Villalobos 2004). Using commercially available oils, Shelly et al. (2004a, b) and Papadopoulos et al. (2006) further demonstrated that the aroma of ginger root oil (*Zingiber officinale* Roscoe) and orange oil conferred a mating advantage to male medflies. Encouraged by these latter results, Shelly et al. (2007a) have promoted the implementation of 'aromatherapy' (large-scale exposure of flies to ginger root oil) to increase the mating competitiveness of medfly males used in sterile release programs.

It is not known why exposure to certain fruits or oils enhances the mating competitiveness of male medflies. Most of the work conducted thus far has involved male exposure to the aroma of ginger root oil (GRO hereafter), and several conclusions can be drawn. Males exposed to GRO spend a greater proportion of their time pheromone-calling than non-exposed males (Shelly 2001; Papadopoulos et al. 2006). However, the relative increase in calling activity appears insufficient to explain the magnitude of the observed mating increase. For example, Shelly (2001) found that GRO-exposed males obtained approximately twice as many matings as non-exposed males, but that the level of pheromone-calling displayed by GRO-exposed males was only about 30% higher than that observed for non-exposed males. Subsequent trials with a wind tunnel confirmed that females were equally attracted to the pheromone of GRO-exposed and non-exposed males (Papadopoulos et al. 2006). Thus, whereas GRO exposure results in a slight increase in male signaling activity, it does not appear to affect the attractiveness of the pheromone signal to females.

The present study further investigates the mechanism that underlies the increased mating success of GRO-exposed males of *C. capitata*. In particular, we present the results of two sets of behavioral experiments that examined the possibility that female preference for GRO-exposed males was mediated by a 'perfume effect', whereby the aroma of GRO affected the male cuticle in some manner that enabled females to distinguish between exposed and non-exposed individuals. In the first set of experiments, we presented females with freshly killed flies or hexane extracts from flies and monitored female presence near the different treatments. By offering such stimuli, we eliminated the possibility that active displays, behavioral or pheromonal, influenced female response. In the second set, we compared mate choice between intact females and females from which one or both antennae were surgically removed. By removing the primary olfactory receptors, we directly examined the role of male odor in affecting female choice. We also conducted the converse experiment, i.e., we compared mating success between non-

exposed males and GRO-exposed males that were intact or in which one or both antennae were surgically removed before the exposure. By performing this surgery, we directly examined the importance of male perception of the GRO odor in influencing mating success.

Methods and Materials

Study Insects Medflies used in this study were derived from a laboratory colony started with >500 adults reared from coffee (*Coffea arabica* L.) berries collected on the island of Kauai, Hawaii. The colony was four to five generations removed from the wild when this study was conducted. Adults of *C. capitata* were held in screen cages and provided a mixture of sucrose and yeast hydrolysate (3:1, *v*/*v*) in a Petri dish, water, and oviposition substrate (perforated plastic vials containing small sponges soaked in lemon juice). Eggs were placed on standard larval medium (Tanaka et al. 1969), and mature larvae 'popped' onto a layer of vermiculite for pupation. Adult medflies used in the experiments were separated by sex within 48 hr of eclosion, well before reaching sexual maturity at 6–8 d of age. When used in the experiments, males were 9–13 d old and females were 10–15 d old. Flies were held in screen-covered, plastic containers (5 l; 125–150 flies per container) at 23–26°C and 60–90% RH and received both natural and fluorescent light on a 12 L/12 D photoperiod. Experimental flies were maintained on the same sugar-yeast hydrolysate mixture used for the colony.

In several experiments, we also used males of the oriental fruit fly, *Bactrocera dorsalis* (Hendel), derived from a laboratory colony started with >200 adults reared from guava (*Psidium guajava* L.) fruits collected on the island of Oahu, Hawaii. The colony was two generations removed from the wild when this study was conducted. The oriental fruit fly colony was maintained in much the same manner as the medfly colony, except that eggs were deposited and larvae developed in fruit (papayas, *Carica papaya* L.). When used in the experiments, *B. dorsalis* males were 22–30 d old (sexual maturation in this species occurs between 15–21 d of age).

Female Response to Dead Flies: Basic Protocol Experiments in which we monitored the response of *C. capitata* females to various treatments of freshly killed flies followed the same basic protocol (with the single exception of experiment 11, see below). On a given test day, 30 females were transferred (with an aspirator) from the holding containers to screen cages (30 cm cubes) between 0830–0900 hours and held (in a different room from the test room) until tested between 1000–1130 hours. Groups of ten 'stimulus' flies from the treatments being compared were transferred from holding containers to a freezer for 15–20 min; flies from the different treatments were placed in separate freezers. Upon removal from the freezer, dead flies were held at room temperature for 10 min, transferred to plastic Petri dishes (5.4 cm diameter) and introduced into the test cages containing the females. Thus, for all trials, a given screen cage contained 30 females and two Petri dishes (10 cm apart), each holding ten freshly killed flies from the two treatments being compared.

Starting 1 min after placement of the stimulus flies, we recorded the number of females resting on each of the dishes at 1-min intervals over a 30-min period. Because females were not marked, the number of sightings recorded represented a composite index that encompassed both female arrival to and retention on a particular dish. An observer

monitored two cages simultaneously (containing the same two treatments of stimulus flies) and one pair of cages per day (with different, randomly selected treatments of stimulus flies in the first and second pairs). During observations, the cages were resting on a table (0.5 m apart) adjacent to a window. For a given experiment, the locations (right or left from the observer's vantage) of the two treatments within the test cages were alternated among the replicates to control for a possible position effect. Females and stimulus flies were used for a single trial only, and the test cages and Petri dishes were rinsed with water at the end of daily observations.

For several experiments, we compared a given pair of treatments first by using uncovered dishes, and then we conducted a second set of replicates by using covered dishes (i.e., a 5 cm square of white cotton cloth over the stimulus flies). By preventing females from seeing or touching the stimulus flies, the cloth cover was used to isolate more completely the role of olfactory cues in influencing female settlement.

Female Response to Dead Flies: Species- and Sex-Specific Attraction We first investigated species- and sex-specific attraction of *C. capitata* females. In experiment 1, females were offered conspecific males and *B. dorsalis* males, and in experiment 2, females were offered conspecific males and females.

Because females showed strongest attraction to conspecific males, we conducted three additional experiments to determine whether conspecific males (not exposed to GRO) had hexane-extractable compounds that were attractive to females. In experiment 3, we monitored female response to paper discs (8 mm diameter) treated with hexane extract from male medflies *vs* discs treated with hexane only. We placed ten chilled males in 0.25 ml of hexane for 1 min, removed them, and then placed a paper disc in the extract until the extract was entirely absorbed. The disc was then dried for 1 hr before testing. The 'control' discs absorbed the same volume of hexane and were dried for 1 hr before use. During the trials, discs were presented in Petri dishes following the standard procedure.

In experiment 4, we compared female sightings between hexane-washed and non-washed males (neither group was exposed to GRO). In this case, washed males were chilled, placed individually in glass beakers that contained 2 ml of hexane, shaken gently for 30 sec, and dried for 1 hr before testing. Because this treatment appeared to remove the volatile attractants, given that hexane-washed males were less attractive to females than non-washed males, we performed experiment 5 to examine the possibility that females were simply avoiding the hexane-washed males. We compared female attraction to two dishes of (non-exposed, uncovered) conspecific males, one of which also contained ten hexane-washed males (obtained following the protocol of the preceding experiment) beneath a cloth cover and the other had only a cloth with no flies beneath (the cloth covers used in this experiment were 2 cm squares, and thus covered only a portion of the dish).

Female Response to Dead Flies: GRO-Exposure to Conspecific Males We next investigated the influence of GRO exposure on the attraction of *C. capitata* females. In experiment 6, we offered females either GRO-exposed or non-exposed conspecific males. To obtain exposed males, we applied 0.25 ml of GRO to a cotton wick, placed the wick in a container (5 l) that held 30 males, and then removed it 2 hr later. Adjusted for container volume, this dose was equivalent to one previously demonstrated to increase male mating performance (Shelly 2001). GRO exposure was conducted in an isolated room between 1000–1200 hours; flies were exposed 1 d before testing (i.e., flies were freeze-killed and bioassayed the following morning).

Because females were more frequently observed near the GRO-exposed than the non-exposed males and given the results of experiment 4, we considered the hexane-wash procedure to be an effective means of extracting compounds responsible for attraction. Therefore, in experiment 7, we repeated the protocol of experiment 6 but (after chilling) extracted the GRO-exposed males with hexane following the procedure described above for experiment 4.

Additional Tests on the Impact of GRO Exposure on Female Response Four experiments were conducted to investigate additional aspects of how GRO exposure affects female attraction.

In experiment 8, we monitored the response of females to paper discs treated with hexane extract from GRO-exposed males *vs* non-exposed males. The GRO exposure and hexane washing procedures followed those described for experiments 6 and 3, respectively.

In experiment 9, we compared female response to conspecific males that had no direct GRO exposure but were confined with GRO-exposed males immediately before testing. This experiment investigated the possibility that the attractant could be transferred among crowded individuals, a phenomenon demonstrated in several *Drosophila* species (Coyne et al. 1994; Marcillac and Ferveur 2004). We placed ten GRO-exposed males (exposure followed the same procedure described for experiment 6, except that in this instance the males were chilled just before GRO exposure and the distal tip of the right wing was clipped for identification) and ten non-exposed males in a vial (*h*/*w*, 5.4 cm, 2.3 cm) for 1 hr. During this interval, there was considerable physical contact among males, presumably allowing for the transfer of scent from the GRO-exposed males to the non-exposed males. The vial was chilled, and the males that were not exposed directly to GRO (those with intact wings) were used in the trials. Males in the other treatment, i.e., those not exposed to GRO-exposed males, were subject to the same confinement protocol, but in this case the 'other' (non-test) males placed in the vial had not been exposed to GRO.

In experiment 10, we assessed whether GRO exposure per se (i.e., independent of the object exposed) resulted in higher female attraction by comparing female attraction between GRO-exposed *vs* non-exposed males of *B. dorsalis*. Protocol followed that described for experiment 6.

In experiment 11, we monitored female response to GRO-exposed *vs* non-exposed males in a more complex environment, i.e., in the canopy of live, potted plants of *Ficus benjamina* L. Observations were made in a glass aquarium (*l*/*w*/*d*, 50×27×30 cm) that contained two plants; the aquarium was oriented 90° from normal (i.e., with a short side resting on the table top) with the open side covered with a screen mesh. For a given trial, we suspended two screen cages (cylinders, *h*/*w*, 5.0 cm, 3.0 cm) in the canopy that contained four GRO-exposed (after the exposure protocol used in experiment 6) or four non-exposed males (males from both groups were freshly killed through chilling), introduced 40 females, and then recorded the number of females resting directly on, or within 3 cm of, each cage at 1-min intervals over 30 min. The cages were placed at the same locations over all replicates, but the type of male (GRO-exposed or non-exposed) placed at a given location was alternated between successive trials. The aquarium, plants, and cages were rinsed with water after each trial.

Mating Trials: Female and Male Antennal Removal Two sets of mating trials were performed. In the first, we manipulated female ability to perceive olfactory stimuli emitted by males, and in the second, we manipulated male ability to perceive the odor of GRO.

In the first set, we presented GRO-exposed and non-exposed males to intact females or females from which either one or both antennae were removed. Although olfactory sensilla may also occur on the mouthparts (as in *Drosophila*, Shanbhag et al. 1999), the antennae are believed to be the primary olfactory structures in *C. capitata* females (Levinson et al. 1987), and removal of both antennae completely eliminates female response to male pheromone (Nakagawa et al. 1973). For surgery, flies were chilled at 4°C, and the entire antenna(e) was cut off with a finger nail clipper under a dissecting microscope. Intact females were chilled only. Treated males were exposed to GRO (following the method described above) 1 d before testing; both GRO-exposed and non-exposed males were chilled and marked 1 d before testing by placing a small dot of enamel paint on the thorax (the chilling procedure had no apparent adverse effects on subsequent male behavior).

In the second set, we presented intact females with non-exposed and GRO-exposed males from which either one or both antennae were removed, i.e., the two male groups differed in the presence or absence of GRO exposure only and were identical with respect to possible effects of 'surgical trauma' associated with antennal removal. In trials conducted in large screen cages (75×90×120 cm), males lacking both antennae were rarely captured in GRO-baited sticky traps (Shelly, unpublished data), and we presume that removal of both antennae effectively prevented olfactory perception in males. Surgery was performed on males 2 d before testing, and GRO exposure and marking were completed 1 d before testing. All handling procedures were identical to those described above.

Mating Trials: Test Procedure Mating trials were conducted in the laboratory by using screen cages (30 cm cubes) under the same ambient conditions noted above. By using an aspirator, we placed 25 males from each treatment category, GRO-exposed or non-exposed, and 25 females into a cage (females were introduced 15 min after the males). Trials commenced between 0730–0800 hr, and mating pairs were collected at 0.5 hr intervals over a 4-hr period. We established three cages on each of 8 days for trials that involved manipulation of female antennal number (corresponding to intact females and females missing one or two antennae) and two cages on each of 10 d for trials that involved manipulation of male antennal number (corresponding to males missing one or two antennae). Because removal of both female antennae inhibited mating dramatically (a result also noted by Nakagawa et al. 1973 and Levinson et al. 1987), we conducted an additional 17 replicates with these females to gain an adequate sample of mated pairs.

Statistical Analyses In analyzing female attraction, two-way analysis of variance (ANOVA) was used to compare samples in experiments that included covered and uncovered stimulus flies (experiments 1, 2, 6, and 7), and a one-way ANOVA (Student's *t* test for two sample comparisons) was used in experiments using uncovered stimulus flies exclusively (experiments 4, 5, 9, 10, and 11) or paper discs (experiments 3 and 8). In all cases, the Tukey test to identify significant pair-wise differences. In analyzing mating frequency, samples were compared with the Student's *t* test. The data met parametric assumptions in most cases; when they did not, a log transformation was performed to normalize the data. Analyses were performed using SigmaStat Statistical Software (version 2.0). Means + 1 SE are given.

Results

Female Response to Dead Flies: Species- and Sex-Specific Attraction In experiment 1, uncovered or covered males of *C. capitata* and *B. dorsalis* were offered to *C. capitata*

females, and both factors—species identity and presentation method (covered or uncovered)—had effects on female visitation (species, $F_{1,44}$=90.0, P<0.001; presentation, $F_{1,44}$=11.1, P<0.01; Fig. 1a). In addition, the interaction term was significant ($F_{1,44}$=7.0, P<0.05). For both uncovered and covered stimulus flies, females were sighted significantly more often near the *C. capitata* males than near the *B. dorsalis* males. Fewer female sightings were made for covered than uncovered medfly males, whereas no effect of the cloth cover was observed for the oriental fruit fly males.

In experiment 2, uncovered or covered conspecific males and females were offered to *C. capitata* females, and both factors—sex and presentation method—had effects on female visitation (sex, $F_{1,\ 44}$=29.8, P<0.001; presentation, $F_{1,44}$=35.8, P<0.001; Fig. 1b). The interaction term was significant as well ($F_{1,\ 44}$=8.4, P<0.01). For both uncovered and covered stimulus flies, there were more female sightings made near the conspecific males than near conspecific females. For both sexes of stimulus flies, there were fewer female sightings near the covered than the uncovered individuals.

Conspecific males appeared to possess hexane-extractable compounds attractive to females. In experiment 3, there were more female sightings made near paper discs soaked in hexane-wash from male medflies (21.3+3.1) than near discs soaked in hexane only

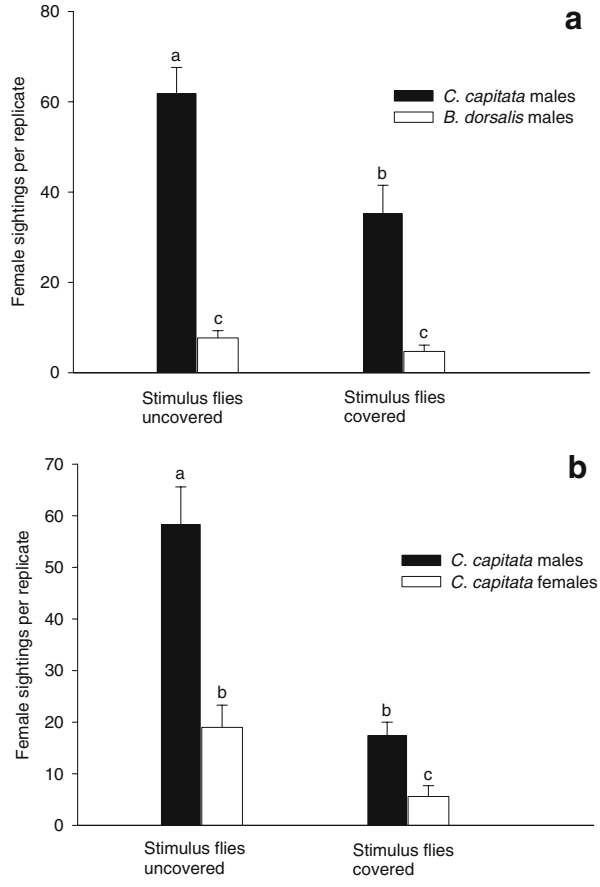


Fig. 1 Sightings of *Ceratitis capitata* females in choice tests near freshly killed **a** males of *C. capitata* and *B. dorsalis* (experiment 1) and **b** males and females of *C. capitata* (experiment 2). *Bar heights* represent mean number (+1 SE) of sightings per replicate (N=12). Means *labeled with same letter* are not significantly different (Tukey test, P=0.05). Note that whereas ANOVA was performed using all sample means, covered and uncovered treatments were presented in separate trials

(10.3+1.8, t=2.4, df=22; P<0.05). In addition, in experiment 4, the number of female sightings recorded for non-hexane-washed males was greater than that recorded for hexane-washed males (85.3+7.5 *vs* 42.1+8.5, respectively, t=2.7, df=22, P<0.05; only trials that used uncovered males were conducted). This difference did not appear to result from female avoidance of the hexane treatment itself. The presence of (covered) hexane-washed males close to non-washed males did not reduce female sightings below that observed for non-washed males presented alone. Female sightings were, in fact, slightly higher for the former group than the latter group, although this difference was not statistically significant (52.8+5.6 *vs* 47.2+4.9 sightings/replicate, t=0.51, df=22, P>0.05, experiment 5).

Female Response to Dead Flies: GRO Exposure to Conspecific Males In experiment 6, uncovered or covered conspecific males that were GRO-exposed or non-exposed were offered to *C. capitata* females, and both factors—GRO treatment and presentation method—had effects on female visitation (GRO exposure, $F_{1,44}$=13.2, P<0.001; presentation, $F_{1,44}$=69.4, P<0.001; Fig. 2a). The interaction term was significant as well ($F_{1,44}$=5.4, P<0.05). For both uncovered and covered stimulus flies, there were more female sightings made near the GRO-exposed males than near the non-exposed males. For both the GRO-exposed and non-exposed stimulus flies, there were fewer female sightings near the covered than the uncovered individuals.

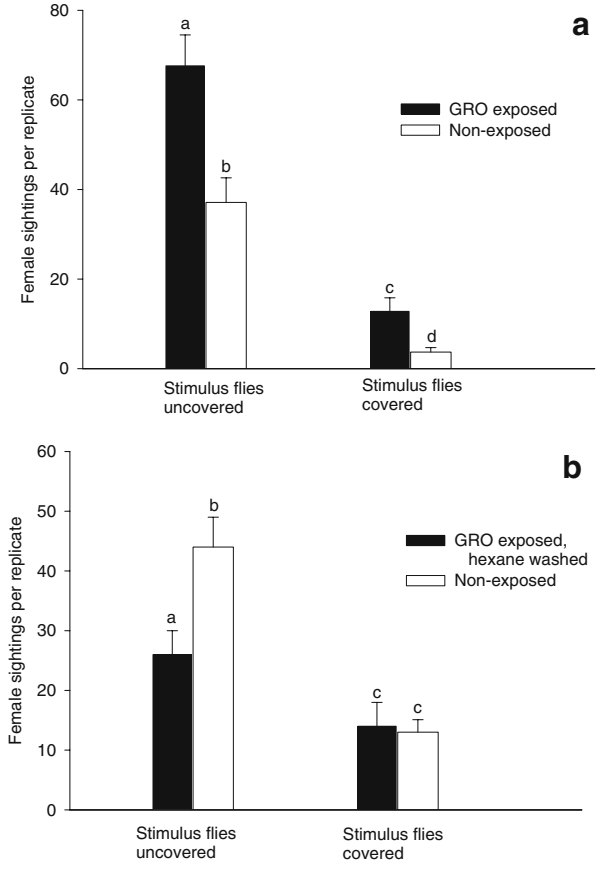


Fig. 2 Sightings of *Ceratitis capitata* females in choice tests near freshly killed males of *C. capitata* that were **a** exposed to GRO or non-exposed (experiment 6) and **b** exposed to GRO and then washed in hexane or non-exposed (experiment 7). *Bar heights* represent mean number of sightings per replicate (N=12); *error bars* represent +1 SE. Means *labeled with same letter* are not significantly different (Tukey test, P=0.05). Note that, whereas ANOVA was performed using all sample means, covered and uncovered treatments were presented in separate trials

Extraction with hexane reduced the attractiveness of GRO-exposed males. In experiment 7, uncovered and covered conspecific males that were GRO-exposed and then hexane-washed or non-exposed were offered to *C. capitata* females, and both factors had effects on female attraction (GRO/hexane treatment, $F_{1,44}=14.4$, $P<0.001$; presentation, $F_{1,\ 44}=6.1$, $P<0.05$; Fig. 2b). The interaction term was significant as well ($F_{1,44}=7.2$, $P<0.05$). For uncovered stimulus flies, there were more female sightings near the non-exposed males than near the hexane-washed, GRO-exposed males. For covered stimulus flies, there was no difference in the number of female sightings for the two male types.

Additional Tests Regarding the Effect of GRO Exposure on Female Response Table 1 presents the results from experiments 8–11. When presented with paper discs that contained extract from hexane-washed males, females were sighted more frequently near discs treated with extracts of GRO-exposed males than non-exposed males (experiment 8). There was also evidence that GRO-induced scent was transferable between males: Females were sighted more frequently near non-exposed males that had been confined with GRO-exposed males than non-exposed males that had been confined with other non-exposed males (experiment 9). Although these tests, along with the previous ones, indicate that GRO has an effect on female response, it does not appear that *C. capitata* females are attracted indiscriminately to GRO-exposed objects independent of their identity. When presented with males of *B. dorsalis*, medfly females were sighted equally near GRO-exposed or non-exposed individuals (experiment 10). When monitored in the larger and more physically complex environment established in the aquaria (experiment 11), females were sighted more frequently near GRO-exposed males than non-exposed males, although this difference was only marginally significant ($P=0.09$).

Mating Trials The first set of mating trials involved manipulation of female antennal number. Both intact females and those lacking one antenna mated preferentially with GRO-exposed males (Table 2). As noted above, females that lacked both antennae showed low mating propensity, and only three matings (one GRO-exposed and two non-exposed males) were recorded for such females over eight cages. Consequently, we set up additional 17 cages with these females, and over all 25 cages, we observed 8 matings with GRO-exposed males and 11 matings with non-exposed males, indicating random choice of mates ($P=0.64$, binomial test).

Table 1 Response of *Ceratitis capitata* females to GRO under varying modes of presentation

Experiment	Mode GRO presentation	GRO treatment	Female sightings per replicate
8	Disc with hexane from medfly males	Exposed	21.2 (4.1)
		Non-exposed	10.3 (1.8)*
9	Medfly males from crowded vials	Exposed	97.3 (7.7)
		Non-exposed	71.4 (4.5)*
10	Oriental fruit fly males	Exposed	6.7 (1.9)
		Non-exposed	7.2 (2.0)**
11	Medfly males in aquarium	Exposed	11.1 (2.5)
		Non-exposed	5.2 (1.4)**

Values represent means (+1 SE) of 12 replicates for experiments 8 and 10 and of 18 and 16 replicates for experiments 9 and 11, respectively. See text for methods used in the individual experiments. Significance level is given for between-treatment comparisons within each experiment, where *$P< 0.05$, **$P>0.05$.

Table 2 Mating trials for *Ceratitis capitata*

	Male treatment		Significance
	GRO-exposed	Non-exposed	
Set 1—Female antennal manipulation (female treatment antennae removed)			
0	12.6 (0.9)	8.6 (0.7)	$P<0.01$
1	12.3 (1.0)	7.8 (0.7)	$P<0.001$
2	8	11	$P>0.05$
Set 2—Male antennal manipulation (male treatment antennae removed)			
1	14.6 (1.1)	8.8 (1.3)	$P<0.01$
2	12.4 (1.0)	8.3 (0.9)	P<0.01

Mean number (SE) of matings per replicate (N=8 for set 1 and N=10 for set 2) are presented with significance level based on the t test, with the exception of the experiment involving removal of both antennae from females where the total numbers of matings are presented over 25 cages, and significance was assessed using the binomial test. In all trials, 25 females and 25 males of each GRO exposure category were placed in screen cages.

In the second set, we manipulated antennal number in males. In these trials, GRO-exposed males achieved significantly more matings than non-exposed males independently of the number of antennae removed from the test males. Among GRO-exposed males, there was no difference in the number of matings obtained by individuals lacking one *vs* two antennae ($P<0.05$). Furthermore, GRO-exposed males accounted for a similar proportion of the total matings in their respective trials (-1 or -2 antennae: 62.8 and 59.9%, respectively, $P>0.05$, test performed using arc sine transformed values).

Discussion

Species- and Sex-Recognition Within the Diptera, profiles of cuticular hydrocarbons, which are primarily contact pheromones with low volatility (Amrein 2004; Vogt 2005), differ among closely related species (e.g., *Drosophila*, Etges and Jackson 2001; *Tabanus*, Hoppe et al. 1990; and *Anopheles*, Carlson et al. 1997) and have an important role in species recognition and reproductive isolation. In contrast, the present study indicates that females of *C. capitata* distinguished conspecific from heterospecific males based on chemical cues associated with volatile compounds. Females displayed species-specific discrimination even when the test males were covered and could not be seen or touched directly. Female medflies were only weakly attracted to *B. dorsalis* males, and their response was independent of the manner in which the heterospecific males were presented (i.e., covered or uncovered). In contrast, females showed a significantly higher attraction to uncovered than covered conspecific males, indicating that visual and/or contact cues influenced female response. Species-specific differences in cuticular hydrocarbon profiles have been reported for larvae of several tephritid species (Carlson and Yocom 1986; Sutton and Steck 1994), and, along with the volatile attractants suggested by the present findings, contact pheromones may also function in species recognition in the Tephritidae.

Our tests also revealed that female medflies use volatile olfactory cues to distinguish sex among conspecific individuals. As with species recognition, *C. capitata* females distinguished conspecific individuals by sex even when these individuals were covered and could not be seen or touched. For both sexes, female attraction was greater for

uncovered individuals, indicating that visual and/or contact chemical cues also influenced female response. Sexual differences in cuticular hydrocarbons have been reported for other Diptera (e.g., *Drosophila*, Suvanto et al. 2000; *Fannia*, Uebel et al. 1977) but, to our knowledge, have not been documented for any tephritid species.

Discrimination Among Conspecific Males Trials that tested female response to freshly killed flies or paper discs indicated that females distinguished GRO-exposed and non-exposed males on the basis of olfactory cues. Even when the males were covered, females were sighted more often near GRO-exposed than non-exposed males, and females preferred to settle near paper discs treated with extract from GRO-exposed than non-exposed males. Washing GRO-exposed males in hexane eliminated female preference for these males, whereas confining non-exposed males with GRO-exposed males increased the attractiveness of non-exposed males, suggesting transfer of substances from the GRO-exposed flies.

Surgical removal of the antennae of females allowed a direct test of the role of olfaction on mate choice. Unfortunately, as noted previously (Nakagawa et al. 1973; Levinson et al. 1987), females lacking both antennae do not mate readily, and we obtained a total of only 19 matings that involved such females. These matings showed no deviation from random choice, with GRO-exposed and non-exposed males, accounting for 42 and 58% of the matings, respectively. Costanzo and Monteiro (2007) reported a similar finding in a nymphalid butterfly, where females with 'blocked' antennae (covered with nail varnish) mated indiscriminately with control males emitting normal odors and treated males whose scent producing glands were blocked and rendered non-functional (see also Pivnick et al. 1992). Based on our results, it appears that the previously noted GRO-mediated increase in male mating success (Shelly 2001; Shelly et al. 2004b) depends on female perception of male-derived olfactory cues. The converse approach—antennal removal from males—showed that male ability to perceive the odor of GRO was not necessary for enhanced mating performance. Males exposed to GRO after removal of both antennae achieved significantly more matings than non-exposed (but antenna-less) males. It thus appears that GRO treatment confers a mating advantage through an external phenomenon, i.e., an alteration in body scent, rather than internal processing, e.g., utilization in pheromone synthesis after absorption of airborne molecules.

Two studies on female attraction to pheromone-calling males further support this interpretation. As noted previously, Shelly (2001), working in a field cage, established leks comprised of GRO-exposed or non-exposed males, respectively, and monitored male calling and female visitation. These observations showed that female visitation was directly related to the calling activity associated with given leks (independent of male type) and, as such, was apparently influenced by signaling level per se independent of signal composition (quality). Additionally, Papadopoulos et al. (2006), working in a laboratory wind tunnel, reported that similar numbers of females arrived to spheres emitting the pheromone odor of GRO-exposed or non-exposed males, respectively. The absence of female preference in this experiment suggests that the increased mating success of GRO-exposed males does not reflect the production of a more attractive, long-range pheromonal signal by GRO-treated males. Instead, it appears that GRO's impact is greatest during close-range courtship.

Briceño et al. (2007) found that GRO-exposed and non-exposed males displayed the same behavioral elements of courtship and that wild (but not mass-reared) females accepted GRO-exposed males, not only more frequently but also more rapidly than non-exposed males. The absence of major behavioral differences between males in the two treatments suggests that other sensory modalities, i.e., auditory or olfactory, may be responsible for the

GRO-mediated increase in courtship success. During courtship, males produce sounds via rapid vibration and rhythmic forward-and-backward movements of the wings (Sivinski et al. 1989), but the significance of such acoustic signals has not been investigated experimentally. Whereas auditory cues cannot be dismissed, the present study supports the hypothesis that females may discriminate among courting males, in part at least, on the basis of GRO-related body scent.

Implications for Understanding Female Mate Selection in Natural Populations

The mating success of male medflies is enhanced through exposure, not only to GRO (Shelly 2001) but also to certain fruits (oranges and guavas; Papadopoulos et al. 2001; Shelly et al. 2004a; Shelly and Villalobos 2004), tree bark (guava, Shelly and Villalobos 2004), and other commercially available oils (e.g., orange oil, Shelly et al. 2004a; manuka oil, Shelly et al. 2007b). Although the active compound is not known with certainty, all these substances (GRO, Takeoka et al. 1990; oranges, MacLeod et al. 1988; Nishida et al. 2000; orange oil, Takeoka et al. 1990; guava bark and fruits, MacLeod and De Troconis 1982; Shelly and Villalobos 2004; manuka oil, Douglas et al. 2004) contain the hydrocarbon sesquiterpene α-copaene, which is known to be a powerful attractant to male medflies (Warthen and McInnis 1989; Flath et al. 1994a,b). Importantly, exposure to α-copaene alone was found to increase the mating success of *C. capitata* males (Shelly 2001). As α-copaene is widely distributed among plants, it appears reasonable to assume that this compound (and possibly other related compounds) influences the mating success of *C. capitata* males in the wild.

One pattern that has emerged from studies of α-copaene-containing substances is that, for the commercially available oils, exposure to the aroma alone is sufficient to boost male performance in *C. capitata*, whereas, for the fruits and bark, physical contact is required for mating enhancement. For example, males exposed to the odor of orange oil enjoy increased mating success, whereas males provided oranges in screened containers (thus preventing contact) gain no mating advantage (Shelly et al. 2004a). This trend may reflect the higher concentration of α-copaene (and related compounds) in the oils than in the plants. The concentration of α-copaene in orange oil is 0.2% (S. Young, personal communication) compared to only 0.7 μg/g for the peel of the fruit (Nishida et al. 2000). Based on these observations, it appears likely that, in the wild, male medflies must touch α-copaene-containing plant structures to gain a mating advantage. We are planning future studies to address whether contact of these structures alone (i.e., without feeding) enhances male mating success.

Adaptive Benefit of Female Preference for Scented Males The adaptive benefit of female preference for scented males remains unknown. In a laboratory study (Shelly 2005), females mated with GRO-exposed males did not lay significantly more eggs or live significantly longer than females mated with non-exposed males. Likewise, whether or not a male was exposed to GRO before mating had no significant effect on the proportion of eggs that hatched or the proportion of eggs that yielded pupae (Shelly 2005). Thus, it does not appear that GRO-exposed males transfer some material resource that directly increases female fecundity or vigor. Alternatively, the emission of particular phytochemically derived scents by males may indicate a superior ability to locate the appropriate plants in the habitat; that is, by selecting males with, presumably, an α-copaene-derived scent over others, female medflies may increase the odds that their sons will have high ability to locate sources of α-copaene and hence enjoy high mating success. This scenario constitutes a case

of runaway selection, whereby female choice provides indirect benefits via a trait that confers an advantage to her sons in sexual competition but is arbitrary with respect to offspring viability (Andersson 1994).

If this runaway selection hypothesis is valid, the honesty inherent in a scent-based signal would appear to vary inversely with the ease with which sources of α-copaene were locatable. In cases where such sources are relatively common, a larger proportion of males might find them by chance alone, and female selection of scented males may be less likely to yield sons with high searching capacity. This situation raises the interesting, more general, possibility that female choice based on exogenously derived, olfactory signals (body scent) may be less reliable than choice based on endogenously produced pheromonal signals, e.g., where mate selection is based on the chemical composition of male pheromone, which may serve as an honest indicator of, for example, developmental stability (Thornhill 1991), immunocompetence (Rantala et al. 2002), or dominance status (Moore 1988).

Acknowledgments We thank Eric Jang, Jocelyn Millar, David Robacker, and Keng-Hong Tan for technical advice. Sylvia Young of Citrus and Allied Essences Ltd. supplied information regarding the chemical composition of ginger root oil. Eric Jang, Jocelyn Millar, and Boaz Yuval provided comments on an earlier draft.

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Introduction

Sponge-feeding nudibranchs of the family Chromodorididae sequester selected chemicals from their prey and reuse them for defense (Karuso 1987; Faulkner 1992; Cimino et al. 1999). Several studies on chromodorid species have suggested a genus-specific distribution of terpenoid secondary metabolites, which are generally furanosesquiterpenes for *Hypselodoris*, diterpenes for *Chromodoris*, and sesterterpenes for *Glossodoris* (Karuso 1987; Faulkner 1992; Cimino et al. 1999). However, this specialization is only a tendency, and it would appear that the various species are not equally specialized (Wahidullah et al. 2006). When specialization evolves, there would likely be intermediate stages from more generalized to more specialized forms. In addition, which metabolites will be found in a given specimen is contingent upon environmental circumstances. Additionally, some confusion in the chemotaxonomy of Chromodorididae has been generated by changes in species names and synonymies. For example, *Chromodoris youngbleuthi*, which has been reported to contain scalarane sesterterpenes (Terem and Scheuer 1986), is currently called *Glossodoris rufomarginata*.

Well-described associations link *Glossodoris* nudibranchs to sponges that contain scalarane compounds (Rogers and Paul 1991; Avila and Paul 1997; Fontana et al. 1999, 2000; Gavagnin et al. 2004). However, sesterterpenoid scalarane metabolites have also been reported from species belonging to the genus *Hypselodoris* (Cimino et al. 1993) and *Chromodoris* (Kernan et al. 1988; Miyamoto et al. 1992, 1999), whereas some *Glossodoris* nudibranchs contain diterpenoid metabolites that exhibit a spongiane skeleton (de Silva and Scheuer 1982; Fontana et al. 1997; Somerville et al. 2006).

The typical sponge metabolite scalaradial (**1**) (Cimino et al. 1974) that contains the biologically significant bis-aldehyde functionality has been described only for *Glossodoris pallida* from Guam (Rogers and Paul 1991). An unidentified epimer of scalaradial has also been reported for *Glossodoris hikeurensis* and *Glossodoris cincta* (Rogers and Paul 1991), whereas metabolites with the scalarane framework but showing different functionalities have been isolated from the other *Glossodoris* nudibranchs.

In particular, it is interesting to note that scalarane sesterterpenes that exhibit the keto function at C-12 have been found in *Glossodoris* nudibranchs (Terem and Scheuer 1986; Fontana et al. 1999; Gavagnin et al. 2004). The 12-keto structural feature is unusual in the scalarane series, and it has been encountered exclusively in *Glossodoris* metabolites, leading to the suspicion that some selected species are able to oxidize the hydroxyl function at the C-12 position of dietary derived scalaranes.

Studies on a population of *G. pallida* from Guam showed that the mollusk is able to sequester scalarane compounds (Fig. 1) from its diet (Rogers and Paul 1991) and to store

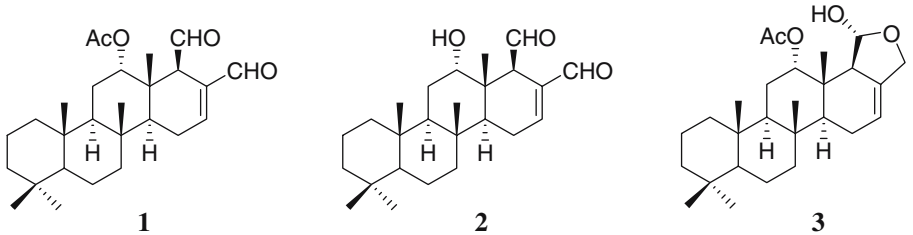


Fig. 1 Scalarane metabolites of *G. pallida* from Guam. Scalaradial (**1**), deacetylscalaradial (**2**), deoxoscalarin (**3**)

them strategically in some selected parts of the body (Avila and Paul 1997). In that work, scalaradial (**1**) (Cimino et al. 1974) and the corresponding deacetyl derivative (**2**) (Yasuda and Tada 1981) were predominant in the mantle dermal formations (MDFs) along the border of the mantle, whereas deoxoscalarin (**3**) was present mainly in the reproductive system/hermaphroditic gland. Ecological experiments demonstrated that the compounds that are localized in the mantle border function as defense compounds against crabs and predatory reef fishes (Avila and Paul 1997). The presence of deoxoscalarin, which was absent in the analyzed sponge prey, was ascribed to the ability of the mollusk to modify dietary scalaradial into a less toxic metabolite by a detoxification process.

In this study, we investigated the chemistry of three *Glossodoris* species from Indo-Pacific areas, *G. pallida* (Ruppell and Leuckart 1828) from China, of *Glossodoris vespa* Rudman, 1990, and *Glossodoris averni* Rudman, 1985 from Eastern Australia. In addition, we re-analyzed the secondary metabolism of a population of *G. pallida* from Guam. Our main purpose was to check the presence of scalaranes in the previously unstudied *G. vespa* and *G. averni* and to verify whether the oxidation of the tricyclic carbon skeleton of scalaradial (**1**) is a peculiarity of *Glossodoris* mollusks.

Methods and Materials

General Procedures Thin layer chromatography (TLC) plates (Merck Silica Gel 60 F254) were used for analytical TLC and Merck Kieselgel 60 for preparative column chromatography. High resolution electron-spray ionization mass spectra (HRESIMS) were carried out on a Micromass Q-TOF micro. HPLC Waters 501 pumps with a refractometer detector were used and equipped with direct-phase column Kromasil Silica, 5 μ (250×4.60 mm, Phenomenex). Nuclear magnetic resonance (NMR) spectra, recorded at the NMR Service of Istituto di Chimica Biomolecolare of CNR (Pozzuoli, Italy), were acquired on a Bruker Avance-400 operating at 400 MHz, in $CDCl_3$ and in C_6D_6 (δ values reported referenced to $CHCl_3$ at 7.26 ppm and to C_6H_6 at 7.15 ppm, respectively) with an inverse probe fitted with a gradient along the *Z*-axis. ^{13}C NMR were recorded on a Bruker DPX-300 operating at 300 MHz (δ values are reported relative to $CDCl_3$, 77.0 ppm and to C_6D_6, 128.0 ppm with a dual probe). Optical rotations were measured on a Jasco DIP 370 digital polarimeter, whereas CD spectra were measured on a Jasco J 710 spectropolarimeter.

Biological Material A single individual of *G. pallida* (size, 5 cm) was collected by scuba diving at a depth of 18 m along the coast of Hainan, in the South China Sea, during September 2004. Animals from the Pacific were collected at a depth of 24 m in Apra Harbor, Guam, at a small patch reef called Sponge Mount, during January 2007. Four individuals (average of 1 cm length) were found on the sponge *Cacospongia* sp., and corresponding sponge pieces were collected with each nudibranch.

Six individuals of *G. averni* (size, 2–7 cm) and seven individuals of *G. vespa* (size, 4 cm) were collected by scuba diving at a depth of 10–16 m along the Queensland coast (Australia) at the Gneerings Reef and at Mudjimba Island offshore from Mooloolaba, during December 2004.

Nudibranchs and sponges from Guam were identified by one of us (P. Schupp, University of Guam), immediately frozen and extracted within a week after collection. The nudibranchs from China and Australia were identified by one of us (E. Mollo, ICB-CNR Italy), immediately frozen and then transferred to ICB in Naples, where they were kept at −80°C until extraction.

Extraction and Isolation Procedures

Glossodoris pallida The single *G. pallida* specimen from Hainan was carefully dissected into the mantle border, rest of the mantle, and internal organs, and these different tissue types were separately extracted with acetone by using ultrasound. The acetone extracts were concentrated in vacuo, and the residual waters were partitioned with diethyl ether. The organic phases were concentrated under reduced pressure giving crude ether extracts for the three anatomical sections: 32, 20, and 111 mg were obtained for mantle border, rest of the mantle, and internal organs, respectively.

The ether extract of the mantle border (32 mg) was subjected to a silica gel column that was eluted with a light petroleum ether/diethyl ether gradient to give the main compound, 12-deacetoxy-12-oxo-scalaradial **4** [12 mg, R_f 0.52 (light petroleum ether/diethyl ether 2:8)] along with a lipid fraction (15 mg). The ether extract of the rest of the mantle (20 mg) gave 1.1 mg of **4** along with a fatty acid fraction (12 mg). The ether extract of the internal organs (111 mg) was purified by silica-gel under the same conditions as above yielding, in order of increasing polarity (TLC with light petroleum ether/diethyl ether 2:8): 12,16-deacetoxy-12-oxo-scalarafuran (**5**, 0.6 mg, R_f 0.96), 19-acetyl-12-deacetoxy-12-oxo-deoxoscalarin (**6**, 25.0 mg, $_f$ 0.71), deoxoscalarin (**3**, 20.0 mg, $_f$ 0.52), 12α,16β-diacetoxy-scalarolbutenolide (**7**, 0.9 mg, R_f 0.50), 12-deacetoxy-12-oxo-deoxoscalarin (**8**, 1.1 mg, R_f 0.47), and 12-deacetyl-12−*epi*-deoxoscalarin (**9**, 1.0 mg, R_f 0.40).

The four *G. pallida* individuals from Guam were not dissected due to their small size (0.236–0.066 g wet weight). Each specimen was extracted three times by soaking in acetone (60 min) and by using ultrasound (1 min each). Acetone extracts were dried under flowing N_2 to give four crude residues A–D (17, 14, 13, and 7 mg, respectively). Four specimens of *Cacospongia* sp. (18–10 g wet weight) were each extracted three with acetone on stirring (60 min each). The acetone extracts were first concentrated in vacuo, and final concentration of the four crude extracts I–IV (441, 400, 304, and 160 mg, respectively) was achieved under flowing N_2.

The nudibranch acetone extract A (17 mg) was purified by silica-gel chromatography (petroleum ether/diethyl ether gradient) to give, in order of increasing polarity, deacetyl scalaradial **2** [2.0 mg, R_f 0.58 (light petroleum ether/diethyl ether 2:8)], scalaradial **1** (3.0 mg, R_f 0.52), and deoxoscalarin (**3**, 1.0 mg), along with a lipid fraction (7.0 mg).

Analogously with the procedure above described, the sponge acetone extract I (441 mg) was purified by silica-gel chromatography (petroleum ether/diethyl ether gradient) to afford, in order of increasing polarity, deacetyl scalaradial (**2**, 13 mg) and scalaradial (**1**, 71 mg), along with a lipid fraction (220 mg).

Glossodoris averni Six specimens of *G. averni* were treated with acetone by using ultrasonic vibration for 2 min. This procedure allows the selective extraction of the metabolites present only in the external part of the mollusk. Subsequently, the animals were extracted again with acetone by using a pestle (internal part extract). Both acetone extracts were concentrated in vacuo, and the residual waters were partitioned with diethyl ether. The organic phases were concentrated under reduced pressure giving crude ether extracts, 40 mg from the external part and 82 mg from the internal part. Careful comparative TLC chromatography in different eluent systems of both extracts showed a similar secondary metabolite composition, so they were combined and processed together. The total ether extract (122 mg) was purified on a silica gel column eluted with a light petroleum ether/diethyl ether gradient to give, in order of elution, 12,16-deacetoxy-12-oxo-scalarafuran (**5**, 1.5 mg), 12-deacetoxy-12-oxo-scalaradial

(**4**, 4.0 mg), 16-deacetoxy-12-*epi*-scalarafuran acetate (**10**, 2.5 mg) [R_f 0.40 (light petroleum ether/diethyl ether 2:8)], and deoxoscalarin **3** (2.5 mg) [R_f 0.38 (light petroleum ether/diethyl ether 2:8)] along with a lipid fraction (80 mg).

Glossodoris vespa Seven *G. vespa* individuals were extracted as described for *G. averni*. The acetone extracts of external and internal parts were concentrated in vacuo, and the residual waters were partitioned with diethyl ether. The organic phases, which were concentrated under reduced pressure, were compared by TLC chromatography showing identical secondary metabolite distribution. Thus, they were combined to give 70 mg of crude extract. This extract was subjected to a silica gel column and eluted with a light petroleum ether/diethyl ether gradient to give 16-deacetoxyscalarafuran **11** [5 mg, R_f 0.62 (light petroleum ether/diethyl ether 2:8)] and 12-deacetoxy-12-oxo-scalaradial **4** (10 mg) along with a lipid fraction (40 mg).

Results and Discussion

The TLC chromatographic analysis of the three anatomical sections of the single specimen of *G. pallida* from Hainan showed a unique secondary metabolite composition. The mantle border extract contained a spot by TLC (R_f 0.52, light petroleum ether/diethyl ether 2:8), which was selectively present in this section, along with the common fatty acid fraction. A preliminary ^{1}H-NMR analysis revealed that the extract contained a single terpenoid component that exhibits signals typical of a scalarane dialdehyde. A TLC comparison with a standard sample of scalaradial (**1**) showed similar chromatographic behavior (R_f scalaradial 0.52, light petroleum ether/diethyl ether 2:8), but NMR analysis of the purified sample (about 38% of ether extract) indicated that it was 12-deacetoxy-12-oxo-scalaradial (**4**), the scalarane dialdehyde previously reported from *G. rufomarginata* (Gavagnin et al. 2004). The extract of the rest of the mantle contained fatty acids and traces of compound **4**, probably due to a contamination from the border, whereas the extract derived from the internal organs was a complex mixture of metabolites at R_f 0.96–0.40 (light petroleum ether/diethyl ether 2:8). Purification of the internal organs extract led to the isolation of an additional six scalarane compounds, which included deoxoscalarin (**3**) (Cimino et al. 1973) and 19-acetyl-12-deacetoxy-12-oxo-deoxoscalarin (**6**) (Gavagnin et al. 2004) as major components. Among the minor compounds, the new scalarane 12,16-deacetoxy-12-oxo-scalarafuran (**5**) and three known components, 12α,16β-diacetoxy-scalarolbutenolide (**7**) (Lu and Faulkner 1997), 12-deacetoxy-12-oxo-deoxoscalarin (**8**) (Fontana et al. 1999; Gavagnin et al. 2004), and 12-deacetyl-12-*epi*-deoxoscalarin (**9**) (Fontana et al. 1999; Fig. 2) were also isolated.

Acetone extracts of *G. pallida* and *Cacospongia* sp. from Guam were analyzed by ^{1}H-NMR spectroscopy and showed similar metabolite patterns for each of the nudibranch individuals and for each of sponge specimens. The chemical profile of the nudibranch extracts was different from that obtained for the Hainan individual, being characterized by three scalarane compounds, whereas the sponge extracts all contained only two of these metabolites. A representative nudibranch extract (17 mg) was subjected to silica gel chromatography and provided scalaradial (**1**) (Cimino et al. 1974), deacetyl scalaradial (**2**) (Yasuda and Tada 1981), and deoxoscalarin (**3**) (Cimino et al. 1973). Compounds **1** and **2** were also isolated from a representative sponge extract (441 mg) by silica gel chromatography, thus indicating their dietary origin in the mollusk. However, the ratio

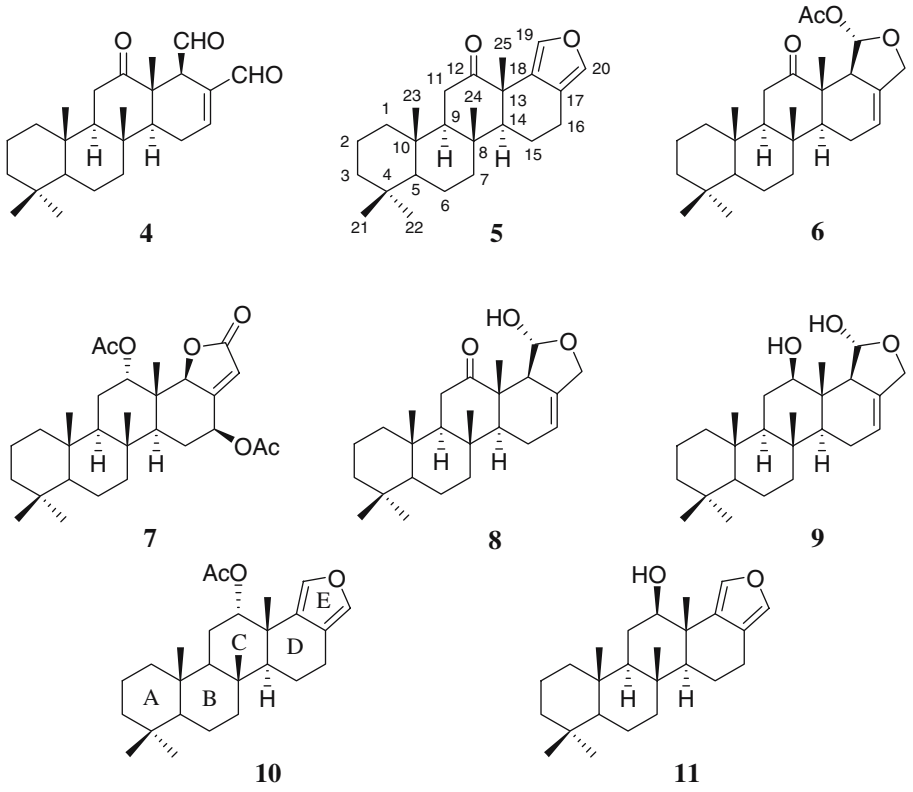


Fig. 2 Scalarane metabolites of *G. pallida* from Hainan and *G. vespa* and *G. averni* from Mooloolaba (compound **3** is not included). 12-Deacetoxy-12-oxo-scalaradial (**4**),12,16-deacetoxy-12-oxo-scalarafuran (**5**), 19-acetyl-12-deacetoxy-12-oxo-deoxosalarin (**6**), 12α,16β-diacetoxy-scalarolbutenolide (**7**), 12-deacetoxy-12-oxo-deoxoscalarin (**8**), 12-deacetyl-12-*epi*-deoxoscalarin (**9**), 16-deacetoxy-12-*epi*-scalarafuran-acetate (**10**), 16-deacetoxyscalarafuran (**11**)

between scalaradial (**1**) and deacetyl scalaradial (**2**) in the nudibranch (**1**:**2**, 3:2) was different from that observed in the sponge (**1**:**2**, 11:2), thus suggesting either distinct accumulation mechanisms or, more likely, that the majority of scalaradial (**1**) is transformed by the mollusk into deoxoscalarin (**3**).

The TLC analysis of the extracts obtained from external and internal parts of *G. averni* revealed similar secondary metabolite patterns. In particular, both extracts contained a mixture of metabolites at R_f values between 0.96 and 0.38 (light petroleum ether/diethyl ether 2:8). After chromatographic purification of the combined extracts, a series of scalaranes (Fig. 2), including the new 12,16-deacetoxy-12-oxo-scalarafuran (**5**), the main compound 12-deacetoxy-12-oxo-scalaradial (**4**) (Gavagnin et al. 2004), 16-deacetoxy-12-*epi*-scalarafuran-acetate (**10**) (De Giulio et al. 1989), and deoxoscalarin (**3**) (Cimino et al. 1973) were isolated along with a lipid fraction.

Analogously with *G. averni*, the TLC chromatographic analysis of the ether extracts of the external and internal parts of *G. vespa* showed a similar composition and distribution of scalarane metabolites. Both extracts were characterized by two main spots on TLC R_f 0.62–0.52 (light petroleum ether/diethyl ether 2:8). Silica-gel purification of the combined extract afforded 16-deacetoxy-scalarafuran **11** (Walker et al. 1980; Tsukamoto et al. 2003) and 12-deacetoxy-12-oxo-scalaradial (**4**) (Gavagnin et al. 2004) along with a lipid fraction (Fig. 2).

The known compounds **1**–**4** and **6**–**11** were identified by comparison of their spectral data (^{1}H-NMR, ^{13}C-NMR, MS, $[\alpha]_D$) with those reported in the literature, whereas the unprecedented 12,16-deacetoxy-12-oxo-scalarafuran (**5**) was characterized as follows.

The molecular formula $C_{25}H_{36}O_2$ was deduced by HRESIMS on the sodiated molecular ion at 391.2618 ($C_{25}H_{36}NaO_2$ requires 391.2613). Analysis of both ^{1}H- and ^{13}C-NMR spectra revealed the presence of a functionalized scalarane skeleton that exhibited a ketone group and a furan moiety. In particular, the ^{1}H-NMR spectrum displayed signals at δ 7.69 (1H, s, H-19) and δ 7.09 (1H, bs, H-20), due to the α-protons of a furan moiety, and at δ 2.78 (1H, dd, J=6 and 16 Hz, H-16a) and δ 2.47 (1H, m, H-16b), attributable to an allylic methylene, which was coupled with another methylene group (H_2-15, δ 1.76, and δ 1.88), in turn linked to an angular methine resonating at δ 1.57 (1H, m, H-14). These data suggested a partial structure (rings D and E), the same as 16-deacetoxy-12-*epi*-scalarafuran-acetate (**10**) and 16-deacetoxy-scalarafuran (**11**), isolated here from *G. averni* and *G. vespa*, respectively. An ABX system at δ 2.68 (1H, t, J=14 Hz), δ 2.41 (1H, dd, J=2.5 and 14 Hz), and δ 1.29 (1H, m) was attributed to the α-keto methylene H_2-11 coupled with the angular methine H-9, thus supporting the location of the keto function at C-12. Finally, as expected for a scalarane skeleton, typical signals for five singlet methyls at δ 1.40 (3H, s, H_3-25), δ 1.10 (3H, s, H_3–24), δ 0.89 (3H, s, H_3–23), δ 0.86 (3H, s, H_3–21), and δ 0.84 (3H, s, H_3–22) completed the proton spectrum. These data indicated the presence of the same partial structure (rings A–C) as compounds **4**, **6,** and **8**. All ^{1}H and ^{13}C NMR resonances of compound **5** (Table 1) were assigned by mono- and bi-dimensional NMR experiments (COSY, HSQC, and HMBC) and were in agreement with the proposed structure. The absolute stereochemistry of compound **5**, which was expected to be the same as that of the natural scalarane series, was further confirmed by comparing the CD profile of compound **5** with that of 12-deacetoxy-12-oxo-scalaradial (**4**), the absolute stereochemistry of which has been determined by synthesis (Gavagnin et al. 2004).

This study has confirmed the typical presence of scalarane metabolites in *Glossodoris* nudibranchs and reinforces the chemical trends reported in the literature for several species belonging to this genus (see Table 2). In addition, the occurrence of scalaranes that exhibit the unusual 12-keto functionality, which has been already reported for other *Glossodoris* nudibranchs, has been observed in the three different mollusks that were investigated. Interestingly, the scalarane content of *G. pallida* from Guam, which was lacking in 12-keto derivatives, displayed significant differences from that of the same species from Hainan.

It is generally assumed that mollusk scalarane metabolites have a dietary origin from sponges belonging to the order Dictyoceratida. The ability of *Glossodoris* nudibranchs to transform selected dietary scalaranes into related compounds as a possible enzymatic detoxification process has been suggested (Rogers and Paul 1991; Avila and Paul 1997; Fontana et al. 2000; Gavagnin et al. 2004). The results obtained in this work on *G. pallida*, *G. averni*, and *G. vespa* seem to confirm this strategy. In fact, 12-keto derivatives, which have never been encountered in sponges, should be obtained by the mollusks by oxidizing the hydroxyl/acetoxyl group located at C-12 in the ring C of dietary scalaranes. Interestingly, similar oxidative modifications that involve rings A or B of the scalarane framework occur in other chromodorid species, such as *Chromodoris funerea* (Kernan et al. 1988) and *Hypselodoris orsini* (Cimino et al. 1993). These mollusks contain a keto-deoxoscalarin with the keto moiety tentatively placed at C-1 or C-3 (**12**) and 6-keto-deoxoscalarin (**13**), respectively (Fig. 3).

A plausible chemical correlation of selected *Glossodoris* scalarane metabolites is depicted in Fig. 4. Deoxoscalarin (**3**) or 12-deacetoxy-12-oxo-scalaradial (**4**) could both be

Table 1 NMR data of compound **5**, 12,16-deacetoxy-12-oxo-scalarafuran

Position	δ $^{1}H^{a,c}$, m, Hz	$\delta^{13}C^{a,d}$	δ $^{1}H^{b,c}$, m, Hz	$\delta^{13}C^{b,d}$
1a	1.64 m	39.1 t	1.34 m	39.1 t
1b	0.82 m		0.54 m	
2a	1.62 m	18.1 t	1.34 m	18.3 t
2b	1.42 m		1.17 m	
3a	1.37 m	42.0 t	1.31 m	42.1 t
3b	1.12 m		1.04 m	
4	–	33.4 s	–	33.1 s
5	0.83 m	56.4 d	1.21 m	56.3 d
6a	1.60 m	18.2 t	1.18 m	18.6 t
6b	1.42 m			
7a	1.97 dt (2.8, 13.0)	41.2 t	1.55 m	41.5 t
7b	1.02 ddd (4.0, 4.2, 13.0)		0.52 m	
8	–	37.5 s	–	37.8 s
9	1.29 m	59.3 d	0.81 m	58.4 d
10	–	38.2 s	–	38.2 s
11a	2.68 t (14.0)	34.8 t	2.32 m	34.9 t
11b	2.41 dd (2.5, 14.0)		2.32 m	
12	–	212.0 s	–	210.0 s
13	–	49.2 s	–	49.0 s
14	1.57 m	57.0 d	0.52 m	56.1 d
15a	1.88 m	17.6 t	1.48 m	17.7 t
15b	1.76 m		1.48 m	
16a	2.78 dd (6.0, 16.0)	20.8 t	2.56 dd (6.0, 16.0)	20.7 t
16b	2.47 m		2.31 m	
17	–	119.6 s	–	119.1 s
18	–	129.4 s	–	129.5 s
19	7.69 s	138.2 d	8.20 s	139.3 d
20	7.09 bs	135.9 d	7.03 s	136.8 d
21	0.86 s	33.1 q	0.84 s	33.7 q
22	0.84 s	21.4 q	0.80 s	21.4 q
23	0.89 s	15.4 q	0.66 s	15.5 q
24	1.10 s	16.9 q	0.76 s	16.6 q
25	1.40 s	26.2 q	1.20 s	26.3 q

[a] $CDCl_3$, chemical shifts (ppm) referred to $CHCl_3$ (δ 7.26) and to $CDCl_3$ (δ 77.0)

[b] C_6D_6, chemical shifts (ppm) referred to C_6H_6 (δ 7.15) and to C_6D_6 (δ 128.0)

[c] By ^{1}H-^{1}H COSY and HSQC experiments

[d] By DEPT, HSQC, and HMBC (J=10 Hz) experiments

obtained from scalaradial (**1**) either by C-20 reduction then formation of the intramolecular acetal linkage, or by hydrolysis then C-12 oxidation, respectively. 12-Deacetoxy-12-oxo-deoxoscalarin (**8**) is formed either by hydrolysis of **3** and subsequent C-12 oxidation of the intermediate compound 12-deacetyl-12α-deoxoscalarin (**14**) or by C-20 reduction of **4**. Finally, the new 12-deacetoxy-12-oxo-scalarafuran (**5**) could be obtained by dehydration and double bond shift of 12-deacetoxy-12-oxo-deoxoscalarin (**8**).

In conclusion, although biosynthetic experiments are necessary to investigate the pathway leading to 12-keto-scalarane derivatives, it seems reasonable to hypothesize that some mollusk species belonging to genus *Glossodoris* have the extraordinary and unique ability to obtain such compounds by enzymatic oxidation of dietary scalaranes. Accordingly, the absence of 12-keto-scalaranes in *G. pallida* from Guam could be explained by the occurrence of geographic variation in the biosynthetic gene expression within the same species, with remarkable evolutionary implications.

Table 2 Scalarane distribution in chromodorid nudibranchs

	1	2	3	4	5	6	7	8	9	10	11	12	13	Other keto scalaranes	Other scalaranes	Diterpenes
Glossodoris atromarginata[a] (from Mandapam-India)								X	X						X	
Glossodoris atromarginata[b] (from Mooloolooba-Australia)																X
Casella atromarginata[c] (=*Glossodoris atromarginata*) (from Sri-Lanka)																X
Glossodoris averni[d] (from Mooloolooba-Australia)			X	X	X					X						
Glossodoris cincta[e] (from Guam)	X														X	
Glossodoris cincta[f] (from Hurghada-Red Sea)																X
Glossodoris dalli[g] (from Costa Rica)			X												X	
Glossodoris hikeurensis[e] (from Guam)															X	
Glossodoris pallida[d] (from Hainan-China)			X	X	X	X	X	X	X							
Glossodoris pallida[d,e] (from Guam)	X	X	X													
Glossodoris rufomarginata[h] (from Hainan-China)			X	X		X		X							X	
Glossodoris sedna[g] (from Costa Rica)															X	
Glossodoris vespa[d] (from Mooloolooba-Australia)				X							X					
Chromodoris funerea[i] (from Kaibakku Lake)			X									X			X	
Chromodoris inornata[j] (from Japan)												X		X	X	
Chromodoris youngbleuthi[k] (=*Glossodoris rufomarginata*) (from Pupukea)		X												X	X	
Hypselodoris orsini[l] (from Gulf of Naples)			X										X		X	

[a] Fontana et al. 1999

[b] Somerville et al. 2006

[c] de Silva and Scheuer 1982

[d] Present work

[e] Rogers and Paul 1991

[f] Fontana et al. 1997

[g] Fontana et al. 2000

[h] Gavagnin et al. 2004

[i] Kernan et al. 1988

[j] Miyamoto et al. 1999

[k] Terem and Scheuer 1986

[l] Cimino et al. 1993.

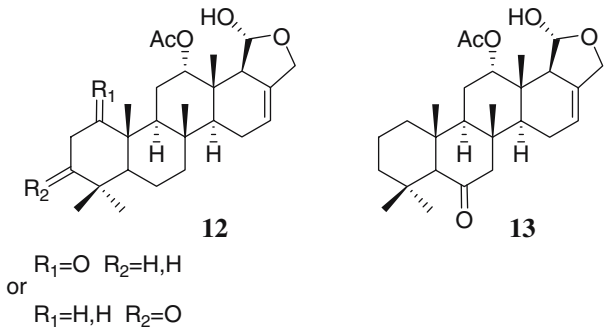


Fig. 3 Structure of keto-deoxoscalarin (**12**) from *C. funerea* and 6-keto-deoxoscalarin (**13**) from *H. orsini*

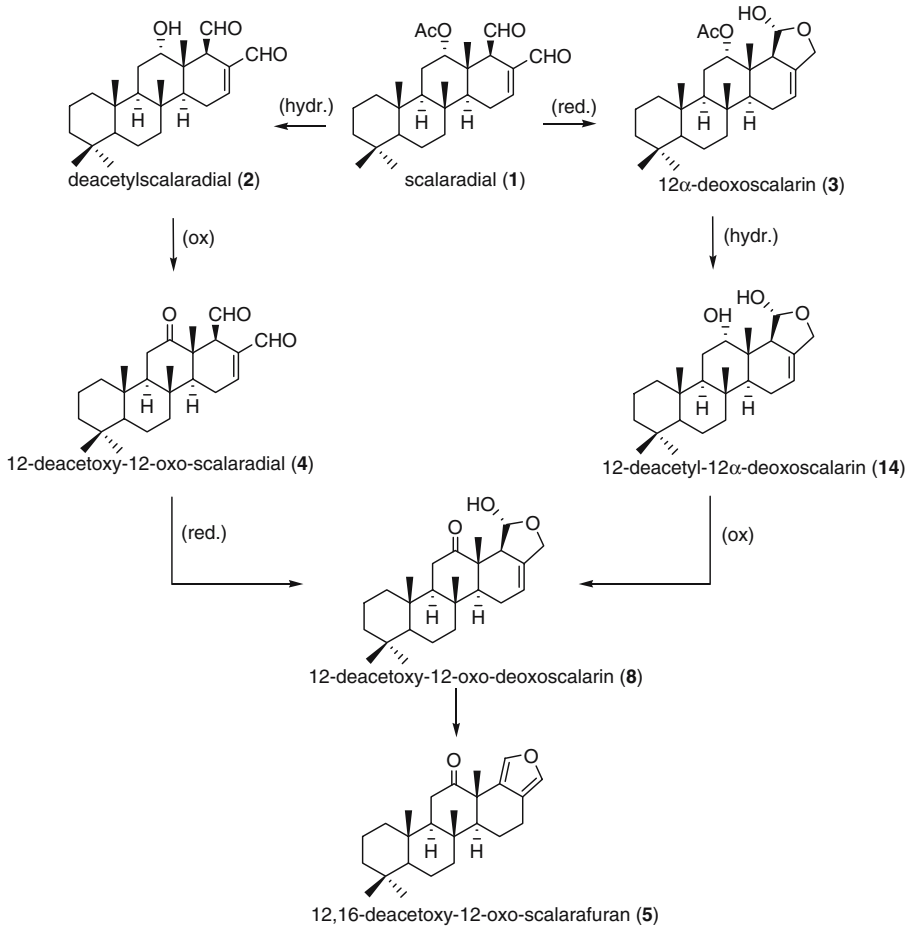


Fig. 4 Chemical correlation of selected *Glossodoris* metabolites

Acknowledgements The authors at ICB are deeply grateful to Mrs. D. Ricciardi for laboratory help. Thanks are due to the ICB-NMR service, in particular to Mrs. D. Melck and Mr. S. Zambardino of the staff service. Mr. R. Turco and Dr. G. Villani are acknowledged for the drawing support and for the antifeedant bioassay, respectively. Mr C. Caballes and Mr. R. Meyers are acknowledged for the assistance in the collection of the Guam specimens. This research was partially supported by the Italian-Chinese bilateral project CNR-CAS "Chemical studies on bioactive molecules with biotechnological interest from Chinese marine invertebrates", the Italian-Australian bilateral project CNR-CSIRO "New bioactives from Mediterranean and Australian seas for control of intestinal nematode parasites of livestock," and the Short Term Mobility-2006 program (CNR, Italy). Y.W. Guo acknowledges the Natural Science Fundation of China (NSFC projects No. 30730108 and No. 20772136). M. J. Garson thanks the Australia Research Council for partial funding. P. J. Schupp acknowledges NIH MBRS SCORE grant SO6-GM-4479615 and SO6-GM-44796–16a for support. This is University of Guam Marine Laboratory contribution number 612.

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different year from most previous studies. Also, large quantities of catechin were detected in blanks for two sampling periods in the present study, leading us to discard those data. This contamination suggests that previous reports of high catechin concentrations that did not include blanks should be viewed with caution. Our results suggest that pure catechin is only rarely present in *C. maculosa* bulk soils. Thus, although catechin may play a role in *C. maculosa* invasion, the infrequency of soil catechin that we determined in this study suggests that we cannot be as certain of its role as previous reports of high soil catechin concentrations suggested.

Keywords Allelopathy · Catechin · *Centaurea maculosa* · *Centaurea stoebe* · Invasion · Novel weapons · Root exudate · Spotted knapweed

Introduction

Centaurea maculosa Lam. (spotted knapweed, recently suggested to be *C. stoebe* L. [USDA, NRCS 2007]) is a Eurasian species in the Asteraceae that invades North American grasslands. It reduces native biodiversity and rangeland forage quality (Watson and Renney 1974; Tyser and Key 1988). A possible role of allelopathy in *C. maculosa* invasion has received considerable attention. Allelopathy has been proposed to mediate some plant invasions because allelopathic exotic plants may produce chemicals to which native species have not yet evolved resistance (i.e., the novel weapons hypothesis) (Callaway and Aschehoug 2000; Callaway and Ridenour 2004). Initial studies of *C. maculosa* allelopathy indicated phytotoxins in *C. maculosa* shoot tissue (Fletcher and Renney 1963; Kelsey and Locken 1987). More recently, an experiment that used activated carbon, which adsorbs organic compounds, suggested that phytotoxins in *C. maculosa* root exudates may inhibit native species (Ridenour and Callaway 2001).

Efforts to identify phytotoxins in *C. maculosa* root exudates revealed a racemic mixture of (±)-catechin (hereafter catechin) (Bais et al. 2002). Since then, several studies have reported high catechin concentrations in *C. maculosa* soils (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006). Catechin also has been reported to inhibit growth and/or reduce survival of susceptible North American plants *in vitro* (Bais et al. 2002, 2003; Weir et al. 2003; Perry et al. 2005a), in soil in controlled environments (Bais et al. 2003), and in soil in the field (Thorpe 2006). (+)-Catechin alone also has been reported to inhibit growth of several plant species *in vitro* (Buta and Lusby 1986; Iqbal et al. 2003; Furubayashi et al. 2007; K. Simoes, unpublished data), but not in one experiment in soil (Furubayashi et al. 2007). (−)-Catechin alone has been reported to inhibit green algae (D'Abrosca et al. 2006). However, catechin effects vary considerably among experimental conditions and species within and among studies (Bais et al. 2003; Weir et al. 2003; Blair et al. 2005; Perry et al. 2005a; Thorpe 2006). Minimum concentrations reported for catechin phytotoxicity range from 0.0016–0.0032 mg g^{-1} (Thorpe 2006) to 0.04 mg g^{-1} (J. Pollock, unpublished data), 0.05 mg ml^{-1} (Weir et al. 2003), 0.25 mg ml^{-1} (Perry et al. 2005a), and >1.0 mg ml^{-1} (Blair et al. 2005). Catechin at high concentrations has been reported to inhibit *C. maculosa* growth and germination, acting as an autoinhibitor (Perry et al. 2005a, b). Finally, catechin has been reported to inhibit the North American species displaced by *C. maculosa* more than European species that coexist with *C. maculosa*, thus providing support for the novel weapons hypothesis (Bais et al. 2003; Thorpe 2006; W. He and Y. Feng, unpublished data).

Other recent studies have reported much lower soil catechin concentrations or have failed to find catechin in *C. maculosa* soil (Blair et al. 2005, 2006). Furthermore, some

studies have reported weak or no effects of high catechin concentrations on the same species for which other studies have reported strong effects (Bais et al. 2003; Weir et al. 2003; Blair et al. 2005; Perry et al. 2005a; Thorpe 2006; W. He and Y. Feng unpublished data; S. Duke personal communication). These results have raised doubts about the role of catechin in *C. maculosa* invasion, and suggested a need for additional research to resolve differences among studies. Therefore, we conducted extensive measurements of soil catechin concentrations. Most previous studies have sampled *C. maculosa* soil at only one time point and at only one or a few sites (Bais et al. 2002, 2003; Blair et al. 2005; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006), with the exception of Blair et al. (2006), who sampled *C. maculosa* soil from July to October. Here, we examined soil catechin in many sites and over a 1-yr time course at one site to better assess spatial and temporal variation in catechin concentrations in *C. maculosa* soils.

Methods and Materials

Sites Soil catechin concentrations were examined at 11 North American sites where *C. maculosa* was abundant (>20% cover; G. Thelen, pers. obs.). Samples were collected during the 2005 and 2006 growing seasons (Table 1). Most sites were sampled only once, but one site (Nelson Gulch 1) was sampled twice and another (Mt. Sentinel) was sampled seven times.

Collection of Soils At each site, 10 1.5-cm diameter×5-cm deep cores were collected from within 5 cm of each of 7–20 randomly selected *C. maculosa* plants with root crown diameters greater than 5 mm. Some sampled plants at the Nelson Gulch site were smaller because *C. maculosa* plants there were generally smaller than at other sites. The cores from around each plant were pooled immediately after collection, sieved through a 2-mm screen, and homogenized by stirring. Two 3-cm^3 subsamples from each homogenized sample were mixed thoroughly with 10 ml of methanol in 15-ml centrifuge tubes. Most samples were placed in methanol immediately in the field. However, samples from Mt. Sentinel on June 14 and July 2, 2005 were placed in methanol 1–2 hr after collection, and samples from Big Creek were placed in methanol 3–4 hr after collection. All samples were stored in methanol at 4°C until processed.

Sample Extraction In preparation for analysis, samples were centrifuged for 5 min at 7,000 rpm (Sorvall SL 50T; relative centrifugal force=5,867×*g*). Supernatants were transferred to new 15-ml tubes, concentrated under blown N_2, resuspended in two rinses of 0.75 ml methanol, transferred to 2.0-ml centrifuge tubes, and centrifuged for 5 min at 14,000 rpm (VWR Galaxy 16; relative centrifugal force=15,996×*g*). The resulting supernatants were transferred to new 2.0-ml tubes, concentrated under blown N_2, resuspended in 0.4 ml methanol, and stored at 0°C until analysis. Extracted soil samples were dried to a constant weight in a 65°C forced-air drying oven and weighed. Soil weights were corrected for 0.06% hydrostatic water determined from weights of a subset of samples dried in a 105°C forced-air drying oven. Catechin concentrations were calculated on a per gram dry soil basis.

Determination of Catechin Concentrations We used methanol to extract soil catechin because we wanted to use methods similar to those with which we had previously measured high catechin concentrations (Bais et al. 2003; Perry et al. 2005b; Thelen et al. 2005; Weir

Table 1 Locations, sampling dates, sample sizes, and soil catechin concentrations for 11 *Centaurea maculosa* sites sampled during the 2005 and 2006 growing seasons

Site	Location	Soil Type[a]	Date[b]	*N*	Soil Catechin (mg g^{-1})		*n*[c]
					Mean±SD	Range	
Skalkaho Pass MT, USA	46.17521°N, 114.04364°W		6/20/2005	20	0	0	0
Clearwater MT, USA	47.00726°N, 113.37319°W	Perma gravelly loam	6/21/2005	40	0	0	0
Blue Mountain MT, USA	46.82703°N, 114.09897°W	Bigarm gravelly loam	6/30/2005	40	0	0	0
Nelson Gulch 1 MT, USA	46.57040°N, 112.14981°W	Windham-Whitecow-Lap channery loams	7/5/2005	10[d]	0	0	0
			8/4/2005	14	0	0	0
Petty Mountain MT, USA	46.97157°N, 114.38408°W		7/7/2005	20	0	0	0
Kooskia ID, USA	46.20869°N, 116.00644°W	Nicodemus loam	7/25/2005	18	0	0	0
Big Creek MT, USA	46.45580°N, 114.18236°W	Dumps, mine	7/29/2005	40	0	0	0
Nelson Gulch 2 MT, USA	46.57047°N, 112.14991°W	Windham-Whitecow-Lap channery loams	8/4/2005	20	0	0	0
Elko BC, CAN	49.29157°N, 115.12134°W		8/6/2005	18	0	0	0
Lee Metcalf MT, USA	46.58737°N, 114.05169°W	Riverside-Tiechute-Curlew complex	8/8/2005	20	0	0	0
Mt. Sentinel MT, USA	46.84102°N, 113.98251°W	Bigarm gravelly loam	6/14/2005	20	0	0	0
			7/2/2005	20	0	0	0
			7/29/2005	20	0	0	0
			9/2/2005	22	0	0	0
			4/6/2006	20	0	0	0
			5/16/2006	20	0.65±0.45	0.14–2.15	20
			6/23/2006	20	0	0	0

[a] Soil information was obtained from the USDA-NRCS web soil survey (http://websoilsurvey.nrcs.usda.gov/app/). Soil information was unavailable for some sites.

[b] The Nelson Gulch 1 site was sampled twice and the Mt. Sentinel site was sampled seven times. All other sites were sampled once.

[c] Number of samples in which catechin was detected.

[d] One subsample, rather than two, was collected from the pooled cores from each plant for this site and date.

et al. 2006). However, methanol extraction results in poor recovery efficiency (<17%) for pure catechin added to soil at known concentrations (Blair et al. 2005; L. Perry, unpublished data). Poor catechin recovery with our method may have caused us to underestimate catechin concentrations, or fail to detect catechin at concentrations lower than our detection limit (0.025 mg g^{-1}).

Catechin concentrations were determined by HPLC in comparison to 1 mg ml^{-1} (±)-catechin standards (Shivambu International, Himachal Pradesh, India). HPLC separations used mobile phase solutions of (A) 1% acetic acid in distilled water and (B) absolute methanol, with a multistep gradient of 0–5 min, 5% B; 5–15 min, increase to 20% B; 15–20 min, 20% B; 20–

40 min, increase to 100% B; 40–50 min, 100% B; 50–55 min, 5% B. The column was a reverse phase, 5 μm C_{18} (4.6×150 mm) (Dionex Corp., Sunnyvale, CA, USA), the flow rate was 1 ml min^{-1}, the sample injection volume was 20 μl, and absorbance was measured at 280 nm. For samples in which catechin was detected by HPLC, the presence of catechin was confirmed by LCMS. LCMS analyses that used mobile phase solutions of (A) 0.1% acetic acid in distilled water and (B) 0.1% acetic acid in absolute methanol, with a multistep gradient of 0–3 min, 10% B; 3–43 min, increase to 90% B; 43–51 min, 90% B. The column was a reverse phase, 5 μm C_{18} (4.6×150 mm) (Dionex Corp., Sunnyvale, CA, USA), the flow rate was 0.7 ml min^{-1}, and absorbance was measured at 280 nm. Ionization for MS analysis was performed in both positive and negative ion modes by using elecrospray ionization with a nitrogen flow of 80 psi, cone voltage of 70 V, needle voltage of 3 kV, and cone temperature of 600°C. Mass data were collected over the range of the gradient program at a rate of one scan per 1.5 sec.

Results and Discussion

In the most extensive study of soil catechin to date, we examined catechin in 402 samples from 11 *Centaurea maculosa* sites. We detected catechin in 20 samples, all collected on one date (May 16, 2006) at one site (Mt. Sentinel, Missoula, MT) (Table 1). No other sites were sampled on that date. The infrequency with which we detected catechin differs from many previous studies of catechin in *C. maculosa* soils in which catechin was detected in most samples regardless of site or date (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006) but is similar to results from Blair et al. (2005, 2006), who reported no catechin or only trace amounts (<0.0001 mg g^{-1}) on eight of 10 dates. The catechin concentrations on May 16, 2006 at Mt. Sentinel (Table 1), however, are comparable to previous reports of high catechin concentrations, which include means of ~2.2±0.2 (Bais et al., 2003), 1.6±0.1 (Perry et al. 2005b), 0.7±0.02 (Weir et al. 2006), and 0.3±0.02 to 3.6±0.1 (± SE) mg g^{-1} (Thelen et al. 2005), and are much higher than those Blair et al. (2006) reported (0.001±0.0004 mg g^{-1}).

There are several potential explanations for the infrequency with which we detected catechin compared to previous studies. Variation in results could be caused by temporal or spatial variation in soil catechin. We detected catechin in every sample from one date at one site, suggesting that specific environmental conditions may be necessary for catechin production, stability, or detection. Conditions could have been poor for catechin in 2005, when little or none was found (Table 1; Blair et al. 2006), compared to 2004 and 2006 when results were mixed (Table 1; Blair et al. 2005; Perry et al. 2005b; Weir et al. 2006), and 2001 when high concentrations were found (Bais et al. 2002, 2003). In the one site studied in multiple years (Mt. Sentinel), high concentrations were reported in 2004 (Perry et al. 2005b), no catechin was detected in 2005, and results were mixed in 2006 (Table 1). Temperature and precipitation did not differ substantially among these years at Mt. Sentinel (Fig. 1), but other factors were not measured. The data from Mt. Sentinel seem to suggest that catechin is present in soil mainly in spring, but other reports from summer and autumn (Bais et al. 2003—June, August; Blair et al. 2006—August; Thelen et al. 2005—November; Weir et al. 2006—July) suggest that it is not predictable with seasons. Thus, temporal variation in conditions such as climate, soil chemistry, microbes, insects, or vegetation might be responsible for variation in soil catechin, but the particular factors that might be important are uncertain. The infrequency with which we detected catechin also

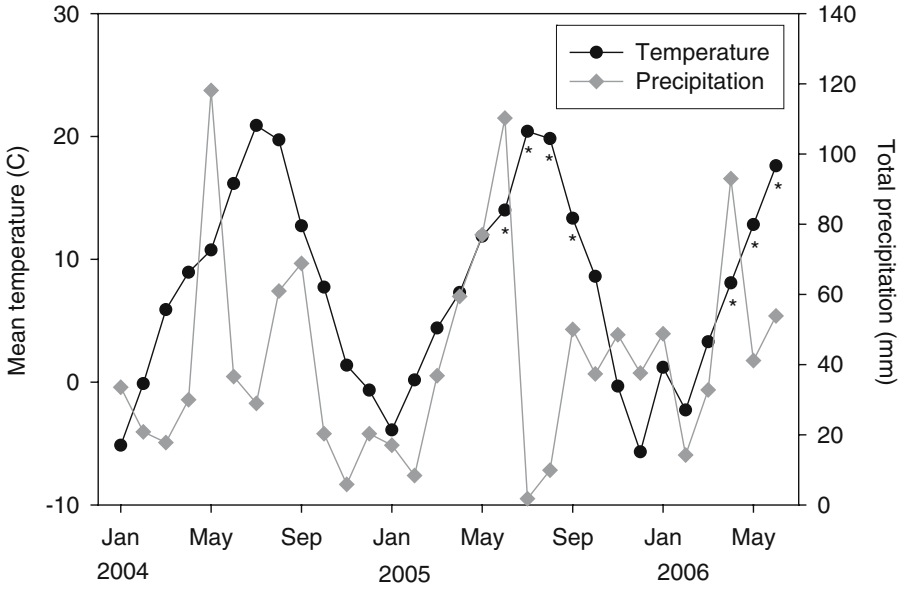


Fig. 1 Climate data for Missoula, Montana from January 2004 to May 2006. Data were obtained from the Western Regional Climate Center (http://www.wrcc.dri.edu). Asterisk indicates months in which samples were collected from Mt. Sentinel, Missoula, MT for the present study (Table 1)

could be explained by spatial variation, with unfavorable conditions in the sites we sampled this time; however, it seems unlikely that 10 of our 11 sites contained unfavorable conditions for catechin, whereas most previous studies sampled favorable sites.

The high catechin concentrations reported previously also could have been caused by undetected catechin contamination. Throughout the present study, we included blanks that contained only methanol as negative controls with our samples during extraction. In one set of samples, we found relatively high catechin concentrations (0.35±0.41 [SD] mg g^{-1}), but also found large quantities of catechin (1.4±0.6 [SD] mg) in all six blanks. In another set of samples, we found catechin in only one sample (0.03 mg g^{-1}), but again found it (0.3 mg) in one of three blanks. Several tests failed to identify the source of the contamination but indicated that it was not an artifact of the HPLC protocol. Because catechin was present in the blanks, we discarded the data for these dates; they are not included in Table 1. In subsequent tests, no quantifiable catechin was found in blanks. However, blanks were not included during sample extraction for most previous reports of high catechin concentrations (Bais et al. 2002, 2003; Weir et al. 2003, 2006; Perry et al. 2005b; Thelen et al. 2005). An exception is Fig. 1C of Thelen et al. (2005), which had one blank. Given the potential for catechin contamination demonstrated by the current study, previous results not including blanks should be regarded with caution.

Differences in our methods between this and previous studies are unlikely to account for our infrequent detection of catechin here. In previous studies, we stored intact soil cores for weeks or months before extracting 0.5-cm^3 subsamples with 1 ml of methanol (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006). In the current study, we sieved and homogenized the soil and extracted 3-cm^3 subsamples with 10 ml of methanol immediately upon collection. Such changes in protocol seem unlikely to have reduced recovery efficiency, particularly to such an extent that we could no longer detect

catechin in most samples. Furthermore, a preliminary experiment in which we compared the two methods suggested that the changes in methods improved catechin detection (data not shown).

The absence of catechin on most dates in the present study and studies by Blair et al. (2005, 2006) suggests that pure catechin (i.e., not complexed with other compounds) may occur only rarely at sufficient concentrations in *C. maculosa* bulk soils to inhibit other plants. Catechin concentrations required to inhibit other plants are uncertain, as minimum concentrations for inhibition vary among studies from 0.0016–0.0032 mg g^{-1} (Thorpe 2006) to 0.04 mg g^{-1} (J. Pollock, unpublished data), 0.05 mg ml^{-1} (Weir et al. 2003), 0.25 mg ml^{-1} (Perry et al. 2005a), 0.27 mg g^{-1} (Inderjit, unpublished data), and weak effects of 1.0 mg ml^{-1} (Blair et al. 2005). Here, our detection limit was 0.025 mg g^{-1}. The detection limits of Blair et al. (2005, 2006) were even lower, 0.00002 mg g^{-1} or trace amounts (<0.0001 mg g^{-1}), much lower even than those Thorpe (2006) reported to be inhibitory. Thus, if pure, stable catechin in bulk soil is an important driver of competitive effects, these results suggest that those effects would have to occur sporadically.

Nevertheless, our results do not disprove a role of catechin or other allelochemicals in *C. maculosa* competitive effects. Studies that have used activated carbon soil amendments, which adsorb organic compounds, indicated that *C. maculosa* root exudates may inhibit growth of neighboring plants (Ridenour and Callaway 2001; Callaway et al. 2005; Perry et al. 2005b). Although catechin does not appear to influence *C. maculosa* ecology via continuously high concentrations of pure catechin in bulk soil, it could play a role through episodic effects on competitors, by producing phytotoxic soil concentrations more frequently, either of phytotoxic catechin-metal complexes (J. Pollock and B. Holben, unpublished data) or phytotoxic degradation products, by occurring more frequently in the rhizosphere or near competing roots than in bulk soil, or by producing frequent but transient catechin pulses (Appel 1993). *C. maculosa* also may produce other phytotoxins with ecological effects. However, none of these hypotheses has been tested.

Although previous consistent reports of high soil catechin concentrations provided circumstantial evidence for a role of catechin in *C. maculosa* invasion (Bais et al. 2002, 2003; Perry et al. 2005b; Thelen et al. 2005; Weir et al. 2006), this more extensive study, together with those of Blair et al. (2005, 2006), suggests that high catechin concentrations rarely occur in *C. maculosa* soil. More confidence can be placed in the current results. Thus, the infrequency of soil catechin weakens the hypothesis that it plays a role in *C. maculosa* invasions.

Acknowledgments This research was supported by the U.S. Department of Defense SERDP (SI-1388 to J.M.V., M.W.P. and R.M.C.). Corey Broekling performed mass spectrometry analyses to confirm the presence of catechin in samples with catechin. Tiffany Weir provided helpful discussions and HPLC troubleshooting. Tom Bassett, Kyle Evans, Courtney Hall, and Nick Wegman assisted with soil sample collection. We thank John Romeo and four anonymous reviewers for comments on previous versions of the manuscript.

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